Investigation of the effect of microwave irradiation (2.45 GHz) on biological systems at constant bulk temperature

By

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Dedication

“This thesis is dedicated to my mother and my father.”

“In loving memory of my grandparents”

Abstract

In this thesis the effect of MW irradiation (2.45 GHz) at constant bulk temperature was investigated on several biological systems.

Studies on enzymatic activity revealed that MW irradiation could enhance the activity of trypsin, however the enzymatic activity of α-amylase and alkaline phosphatase towards the hydrolysis of starch and 4-nitrophenyl phosphate was not affected.

We found that the incorporation of a BODIPY fatty acid (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid) into the cell membrane of PC-3 (human prostate cancer) cells was facilitated by microwave treatment at constant temperature. Also, microwave treatment while non-apoptotic, significantly increased the rate of reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by PC-3 cells. Further studies revealed that MW irradiation (10 W, SAR: 700 mW/ml) could significantly increase the uptake of an anticancer drug (doxorubicin) by PC-3 and MCF-7 (human breast cancer) cells at constant bulk temperature.

Studies on bacterial growth revealed that MW irradiation could significantly decrease the growth of *Escherichia coli* at constant bulk temperature and the impact appeared to be transient. A 2D-gel electrophoresis-based proteomic analysis revealed that the expression

of a series of proteins likely involved in metabolism was affected by the exposure to the MW irradiation.

Overall, the results demonstrated in this study provided additional evidence for the "microwave-specific effects" that are capable of altering the behaviour of biological systems in a way that is quite different from conventional heating (through conduction). Appropriate interpretations of these observations have to also consider the possibility of local heating and/or micro-hotspot formation during MW irradiation. In this respect, the "microwave-specific effects" may not be interpreted either entirely or partially as non-thermal in nature.

Acknowledgments

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My biggest thanks to my parents to whom I owe everything.

Organization of Thesis

This thesis was written in a manuscript-based style. At the time of writing, work from Chapters 3–5 were published, and that from Chapter 6 was submitted for publication. The structure of this thesis is outlined below. Chapters 3–6 are presented in journal-specific formats in their published forms.

Chapter 1: Introduction and literature review. This chapter provided a review of the literature pertaining to the work described in this thesis.

Chapter 2: Microwave radiation accelerates trypsin catalyzed peptide hydrolysis at constant bulk temperature. The project described in this chapter was initially started by Mr. Ben DeLong, but taken over and completed by me. All experiments reported in this chapter were performed by me. This work was published as follows: Mazinani, S. A.; DeLong B.; Yan, H. Microwave radiation accelerates trypsin-catalyzed peptide hydrolysis. *Tetrahedron Lett*. **2015**, *56*, 5804.

Chapter 3: Increase of enzymatic activity by microwave irradiation at constant bulk temperature is enzyme-dependent. Work in this chapter was carried out entirely by me, and has been published as follows: Mazinani, S. A.; Yan, H. Impact of microwave irradiation on enzymatic activity at constant bulk temperature is enzyme-dependent, *Tetrahedron Lett*. **2016**, *57*, 1589.

Chapter 4: Microwave irradiation of PC3 cells at constant culture temperature alters the incorporation of BODIPY into cells and reduction of MTT. Work described in this chapter was carried out in collaboration with Dr. Jeff Stuart's lab. I carried out all the work described in this manuscript, including cancer cell cultures, cell-based assays, confocal microscopy, and flow cytometry. This work was published as follows: Mazinani, S. A.; Moradi, F.; Stuart, J. A.; Yan, H. Microwave irradiation of PC3 cells at constant culture temperature alters the incorporation of BODIPY into cells and the reduction of MTT, *ChemistrySelect*, **2017**, *2*, 7983.

Chapter 5: Microwave-assisted delivery of an anticancer drug to cancer cells. This work was carried out in collaboration with Dr. Jeff Stuart. I performed all the work described in this manuscript, including cancer cell cultures, cell-based assays, confocal microscopy, and flow cytometry. This work was published as follows: Mazinani, S. A.; Stuart, J. A.; Yan, H. Microwave-assisted delivery of an anticancer drug to cancer cells. *RSC Advances*, **2018**, *8*, 31465.

Chapter 6: Exposure to microwave irradiation at constant culture temperature slows the growth of *Escherichia coli* DE3 cells, leading to modified proteomic profile. This work was carried out in collaboration with Dr. Jens Coorssen (2-D gel electrophoresis) and Dr. Stephanie Cologna's (mass spectrometry) labs. I performed bacterial culture experiments, including establishment of growth curves, determination of dissolved oxygen level, cell harvesting, and I assisted with protein isolation and early work in 2D-gel electrophoresis. Work from this chapter has been submitted for publication with *RSC Advances*.

Chapter 7: Summary and conclusion. This chapter provided a summary of the work described in this thesis. A synopsis of future directions of research was also outlined.

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Abbreviations

2DE	Two-dimensional gel electrophoresis
APC	Allophycocyanin
BAPNA	*N*α(±)-benzoyl-D/L-arginine 4-nitroanilide hydrochloride
BODIPY	Boron-dipyrromethene (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene)
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
DMEM	Dulbecco's Modified Eagle Medium
DO	Dissolved oxygen
Em	Emission
EM	Electromagnetic
EMF	Electromagnetic field
eV	Electron volt
Ex	Excitation
GHz	Giga Hertz
h	Hour
Hz	Hertz
IR	Infra-red
MHz	Mega Hertz
min	Minute
MTT	Methylthiazolyldiphenyl-tetrazolium bromide- (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

MW	Microwave
nm	nanometers
PI	Propidium iodide
PS	Phosphatidylserine
rcf	Relative centrifugal force
RF	Radiofrequecy
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAR	Specific Absorption rate
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV	Ultra violet
VGCC	Voltage gated calcium channel
WHO	World health organization

Chapter 1

Introduction and literature review

1.1. Introduction

Modern human is under ever increasing exposure to a variety of low frequency electromagnetic radiations including microwaves (MW). However, it is still unknown if such exposures pose risks to human health. The existence of "microwave-specific effects" has been a controversial issue over the past decades. While some researchers have attributed the reported impacts solely to the heating properties of microwave irradiation (see Szabo et al., 2003; Zhadobov et al., 2007; Zhadobov et al., 2009; Kappe et al., 2013; Reddy et al., 2013 for example), others concluded that differences observed in systems exposed to microwave irradiation and conventional (through conduction) heating are best explained as non-thermal in nature, or as a result of the unique, "microwave-specific", property of microwave as a heating source (see Banik et al., 2003; Belyaev 2005; Sawada & Yamada, 2018 for example). With regards to the reported effects attributed to microwave irradiation, concerns have been raised over the use of relatively high microwave power output, temperature measurements not being carried out directly in the system of interest itself, and/or temperature rises of the system under irradiation not being adequately regulated, leading to difficulty in the interpretation of results. Various effects of MW irradiation on a variety of different systems including health related effects have been reported to date. In fact, the World Health Organization (WHO) classified radiofrequency radiation, including microwave radiation, as a possible carcinogen (Group 2B) in 2010 (WHO, 2010). Considering the ever-increasing exposure

of modern humans to MW irradiation, a better understanding of the potential impacts of such irradiation on biological systems is crucial.

In this thesis attempts were made to meticulously control the conditions, especially the temperature, of the system of study, while being MW irradiated at relatively low energy output (not above 20 W), in each experiment. In these series of studies, the temperature of the system of study was maintained within a few (±2°C) degrees of Celsius from the setpoint. It is expected that the use of relatively low energy output, together with consistent temperature control, will likely limit the scope of heat accumulation at local environments, and thus enable more meaningful interpretation of results.

1.2. Electromagnetic radiation (EM)

Electromagnetic radiation is a stream of photons that propagates through space or any medium in the form of waves. These waves consist of two oscillating electric and magnetic fields which are perpendicular to each other and to the direction of propagation as shown in Figure 1.1. EM waves are categorized based on their frequency or wavelength.

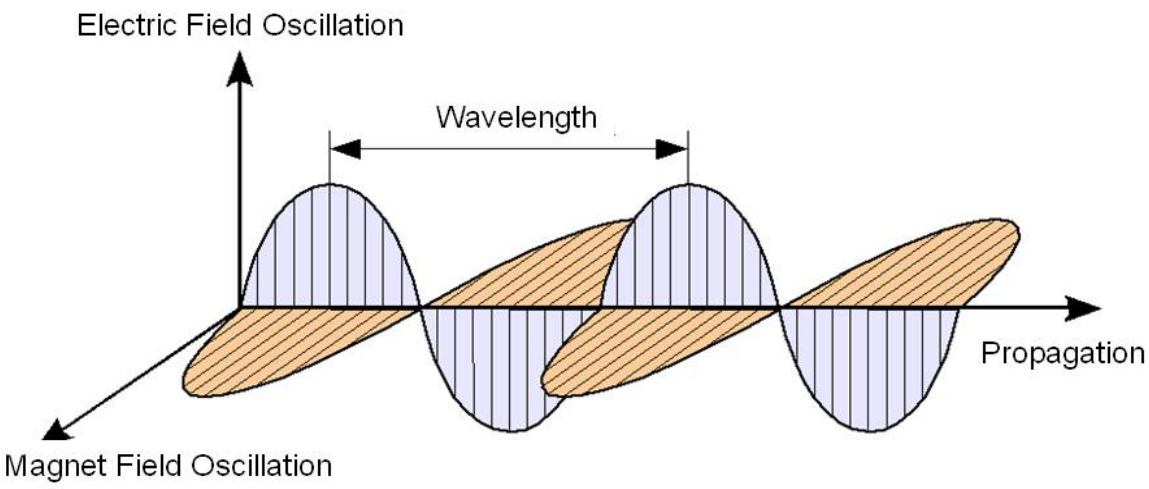

Figure 1.1: Electromagnetic wave. Image obtained from https://depts.washington.edu/cmditr/modules/lum/electromagnetic_radiation.html.

Microwaves are types of EM waves that have a frequency within the range of 300 MHz to 300 GHz. Since the speed at which an electromagnetic wave propagates through empty space or any other homogenous medium is constant, by knowing the frequency of an EM wave, its wavelength can be determined through Equation 1.1.

$$\lambda = C / \nu \qquad (1.1)$$

where C is the speed of propagation of an EM wave (299,792,458 m/s in vacuum), λ (lambda) is the wavelength and ν (small letter nu) is the frequency of the EM wave. Using Equation 1.1, microwaves can be defined as the portion of EM waves that have a

wavelength of approximately 1 millimeter to 1 meter in empty space. At the frequency of 2.45 GHz (used in this study), the wavelength of microwave is approximately 12.2 cm.

1.3. Ionizing and non-ionizing radiation

Electromagnetic radiation is divided into two categories, ionizing and non-ionizing radiation, depending on the frequency or wavelength of the radiation which determines the energy level of their photons (Figure 1.2). Non-ionizing radiation has longer wavelength and lower energy levels compared to the ionizing radiation. Radiofrequency, microwave, infrared, visible and near UV radiation are examples of non-ionizing radiation. Unlike ionizing radiation, photons of non-ionizing radiation are not energetic enough to remove an electron from an atom and cannot cause ionization of a molecule. It is possible to calculate the energy level of a photon using Planck's equation (Equation-1.2)

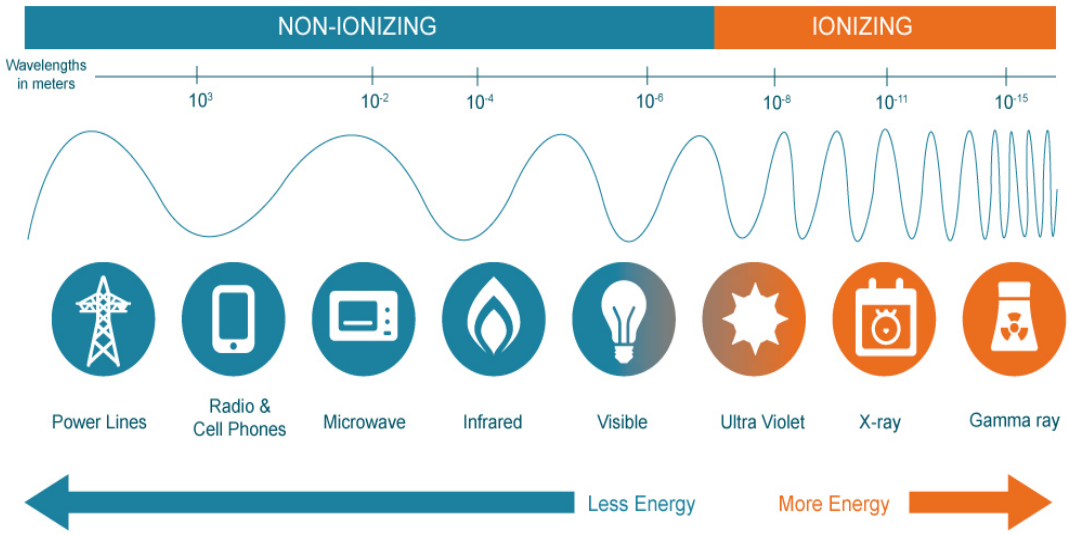

Figure 1.2: Electromagnetic radiation spectrum. Image obtained from https://www.mirion.com/introduction-to-radiation-safety.

$$E = h\,\nu \qquad (1.2)$$

where E is the energy of the photon in Joules (J), h is the Planck's constant which is 6.626×10^{-34} J·s and ν (small letter nu) is the frequency of the photon in s^{-1} or Hertz (Hz). Using Equation 1.2, the energy of a photon of microwave radiation with frequency of 2.45 GHz (which is the frequency used in this study) is calculated to be 1.62×10^{-24} J (≈ 1.01 x 10^{-5} eV as one electron volt (eV) is ≈ 1.6×10^{-19} J). This level of energy is several orders of magnitude below that of chemical bonds as can be seen in Table 1.1.

Table 1.1: Examples of chemical bond energies in electron volt (eV). Microwave photons are not energetic enough to break even weakest types of chemical bonds.

Chemical Bond	Energy (eV)
C-C	3.61
C=C	6.35
C-O	3.74
C-H	4.28
O-H	4.80
Hydrogen Bond	0.04-0.44

(Obtained from: Kappe et al., 2012. Microwaves in Organic and Medicinal Chemistry. Note: Breaking hydrogen bonds does not cause ionization.)

1.4. Research instrument

A CEM Discover Coolmate microwave system functioning at 2.45 GHz was used in the studies reported in this thesis. This system is composed of two main parts; CEM Discover which microwave irradiates the reactants in the reaction vessel and Discover Coolmate which circulates a cooling fluid (perfluoropolyether Galden HT 110) that has negligible specific absorption rate (under microwave irradiation) in the cavity (cooling jacket) circumventing the reaction vessel (Figure 1.3.). The coolant can be pre-cooled in a reservoir by either dry ice or liquid nitrogen A fiber optic probe (CEM Part Number 314307, range -50 °C to 250 °C) inserted in the reaction vessel is used to monitor the temperature in real time (Figure 1.4). The system provides the ability to keep relatively constant bulk reaction temperatures while the reaction mixture is exposed to microwave irradiation. The temperature read from the fiber optic provides feedbacks to the system so that the microwave output (which can be set to not exceed a defined level in Watts) can be adjusted in a real-time fashion to maintain a pre-determined temperature in the reaction mixture. In CEM Discover, the reactants are exposed to a radiative near-field (Fresnel region) electromagnetic field at 2.45 GHz. The specific absorption rate of 2 ml of water under microwave irradiation at 10 W (which was the maximum set-value in the majority of the experiments performed) was calculated to be 700 mW/ml. In addition to the power output, which is automatically adjusted, the flow rate of the cooling media can also be manually adjusted to increase or decrease the rate of cooling. To ensure thermal homogeneity in the reaction vessel, a stirring bar (1×8 mm) was used at either 800 or 540 rpm.

It is worthwhile mentioning that the level of SAR used in the study is dramatically higher than the level considered safe for human exposure as reported in Health Canada's Safety Code 6. The SAR averaged over whole human body mass exposed to electromagnetic radiation in the frequency range of (100 kHz – 6 GHz) should not exceed 0.4 W/kg in a controlled environment and 0.08 W/kg in an uncontrolled environment (Health Canada, 2015). Also, the SAR averaged for the head, neck and trunk should not exceed 1.6 W/kg and 8 W/kg in uncontrolled and controlled environments respectively. The SAR averaged in the limbs should not exceed 20 W/kg in a controlled environment and 4 W/kg in an uncontrolled environment (Health Canada, 2015). A controlled environment is defined as an environment in which the radiofrequency (RF) field intensities have been adequately characterized by means of calculation or measurement and exposure is incurred by persons who are aware of the potential for RF field exposure, cognizant of the intensity of the RF fields in their environment, aware of the potential health risks associated with RF field exposure and able to control their risk using mitigation strategies (Health Canada, 2015). An uncontrolled environment is an area in which any of the criteria defining the controlled environment is not met (Health Canada, 2015).

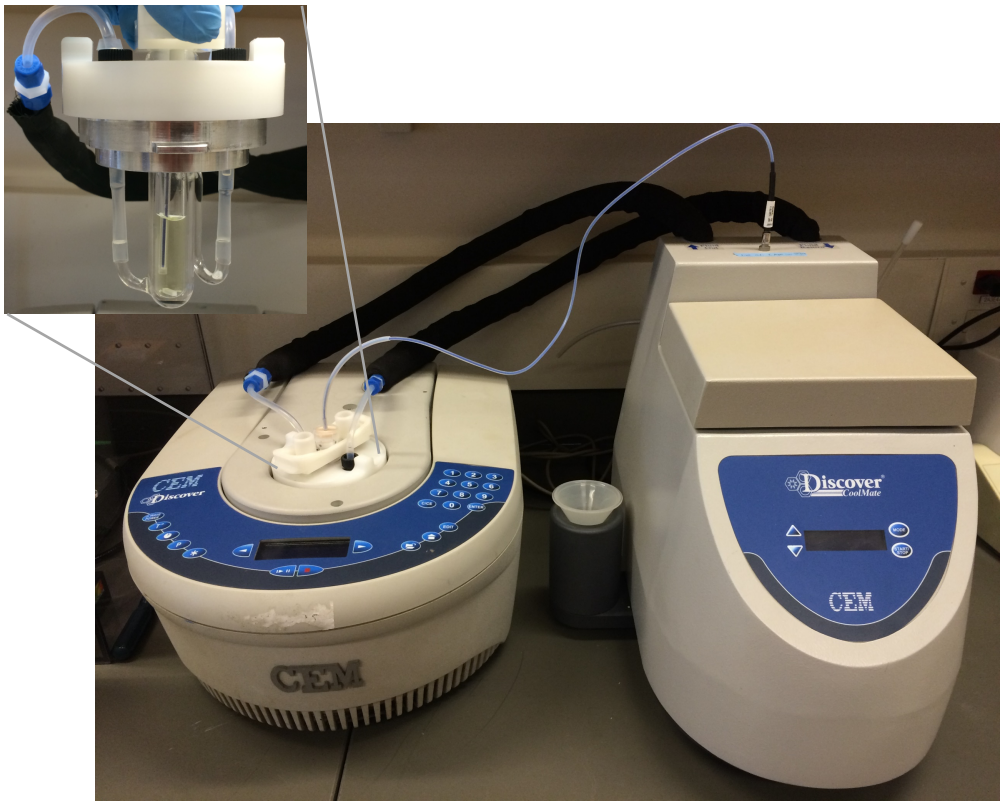

Figure 1.3. CEM Discover microwave reactor (left) and Discover Coolmate (right).

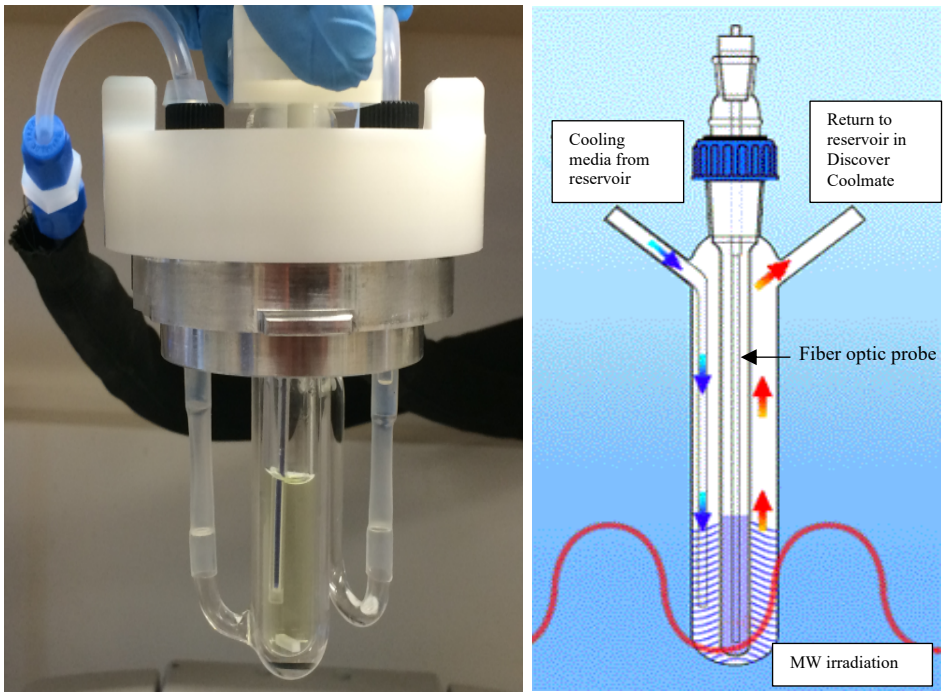

Figure 1.4. Photo of the cooling jacket containing the reaction vessel (left) and schematic of the reaction vessel (right). Schematic obtained from http://www.cem.com/coolmate with modification.

1.5. MW interaction with biological systems - mechanistic considerations of microwave-specific effects

Impact of microwave irradiation at different frequencies and powers on various biological systems has been studied quite extensively. Microwave irradiation can be absorbed by molecules and thereby increase their kinetic energy to some extent, exerting a thermal effect. Since separation of thermal effect of microwaves from any potential non-thermal effects is nearly impossible while irradiating a system, the term "microwave-specific effect" is suggested by the scientific community to describe the unique effects of microwave on biological systems that cannot be reproduced by conventional (through conduction) heating methods. Therefore, these effects are not necessarily strictly non-thermal. Studies are divided on whether a microwave-specific effect exists or not.

One of the common issues with many studies reporting a microwave-specific effect is the difficulty in the regulation of the temperature of the system being studied. Some studies relied solely on external infra-red thermometers (placed outside the reaction vessel) which are believed to not be as accurate as fibre-optic probes in measuring the actual temperature of reactants inside the reaction vessel under microwave irradiation. External infra-red probes measure the temperature of the glass surface of the reaction vessels while fibre-optic probes measure the temperature of the solution inside the reaction vessel. Obermayer and Kappe compared temperature readings of a fibre-optic probe with an infra-red sensor in a CEM Discover LabMate single-mode instrument while microwave irradiating (2.45 GHz, variable power up to 100 W) 3 mL samples of 1-butyl-3-

methylimidazolium bromide (bmimBr) in a series of experiments. Comparison of the temperature profiles obtained by the fibre-optic probe and the infra-red sensor revealed substantial differences between the two, with gaps as large as 20 °C. The authors suggested that the temperature readings of external infra-red probes can be significantly different from internal fibre-optic probes specially when applying microwave irradiation at high (e.g. >50 W) powers (Obermayer & Kappe, 2010). Therefore, the bulk temperature of a system of study could be substantially different from the temperature used in control conditions in studies that relied solely on external infra-red probes.

A number of studies have investigated the potential causes of influences of microwave exposure on biological systems. Among these, calcium flux and generation of reactive oxygen species are discussed below.

1.5.1. The effect of microwave irradiation on voltage gated calcium channels

Calcium is one of the major ions involved in cell functions and its concentration is highly regulated inside the cells partly via voltage gated calcium channels (VGCCs). Calcium ions can act as second messengers and are involved in multiple signal transduction pathways. Cytosolic free calcium (Ca^{2+}) concentration regulates various cellular processes such as cytoskeletal rearrangement, proliferation, differentiation, gene expression and cellular metabolism (Carey & Matsumoto, 1999; Foreman et al., 2006; Rao et al., 2008). Some studies have reported that MW irradiation activated VGCCs leading to increased influx of calcium ions and subsequently causing a variety of biological effects (Rao et al., 2008; Pall, 2013; Pall, 2014). Some of the reported MW effects through VGCCs, which are hypothesized to be involved in cancer (Figure 1.3),

include increased intracellular nitric oxide level, anti-apoptotic effects, changes in cell morphology and proliferation and increased production of reactive oxygen species (Rao et al., 2008; Pall, 2013; Buchanan et al., 2016). Rao et al. investigated the effect of MW irradiation at various frequencies within the range of 700 to 1100 MHz on neuronal cells derived from mouse P19 embryonal carcinoma cells. The authors found statistically significant increases in the number of Ca^{2+} spikes (transient increases in the cytosolic Ca^{2+} levels) in the neuronal cells exposed to MW irradiation with a peak at 800 MHz, with a specific absorption rate (SAR) of 0.5-5 W/kg. Further investigation by the authors revealed that the treatment of cells with nifedipine and ω-conotoxin GVIA peptide, which are potent VGCC inhibitors, could severely suppress the MW-induced Ca^{2+} spikes in the neuronal cells (Rao et al., 2008). In several studies, it was found that calcium channel blockers could block or significantly reduce majority of the observed EMF effects on cells, suggesting that many of such effects were mediated through VGCCs (Papatheofanis, 1990; Rao et al., 2008; Pall, 2013). However, opposing views also exist and some studies did not detect a significant change in intracellular calcium ion concentration during MW exposure on three different cell types, human endothelial cells, rat neuroblastoma (PC-12) and primary rat hippocampal neurons (Schwartz et al., 1993; O'Connor et al., 2010). O'Connor et al. investigated the effect of MW irradiation on the mean intracellular Ca^{2+} concentrations in a few cell types at 900 MHz with SAR of 0.012-2 W/kg, but did not detect a significant change in any of the cell types tested (O'Connor et al., 2010).

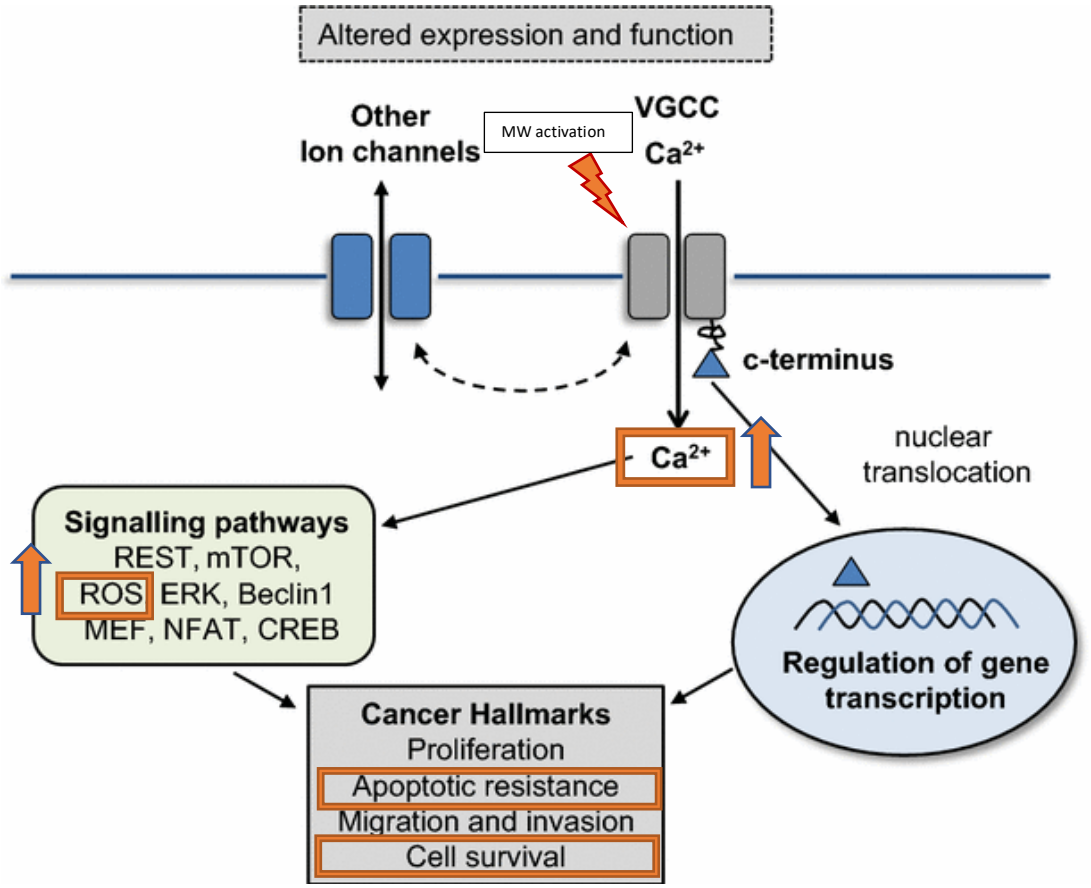

Figure 1.3: Schematic of potential activation of VGCCs by MW irradiation and potential mechanisms of VGCCs in cancer through calcium ion-dependent and independent signalling pathways. A fragment produced by the proteolytic cleavage of c-terminus of VGCCs, translocates to the nucleus and regulates the transcription of oncogenes. Image obtained from Buchanan et al., 2016 with modification.

1.5.2. The effect of microwave irradiation on the intracellular level of reactive oxygen species

Several studies have reported that low intensity MW irradiation of cells increased intracellular levels of reactive oxygen species (ROS) leading to oxidative damage (Yao et al., 2008; Xu et al., 2010; Campisi et al., 2010). ROS at low concentrations can have beneficial effects to cells such as providing protection against infectious agents. However, its excessive production can lead to cellular damage. ROS can damage membranes, proteins and nucleic acids. For instance, ROS can react with guanine to form a highly mutagenic and carcinogenic 8-oxoguanine (Marjanovic et al., 2012).

Yao et al. investigated the effect of MW irradiation at 1.8 GHz on the intracellular ROS levels of human lens epithelial cells (HLECs). The authors found a significant increase in the intracellular ROS levels of HLECs after exposure to MW irradiation (1.8 GHz, SAR 2-4 W/kg) for two hours (Yao et al., 2008). In another study, Campisi et al. found a significant increase in the intracellular ROS levels of rat neocortical astrocytes exposed to MW irradiation (900 MHz, SAR 0.25 W/kg, 20 min), while no change in the cellular viability, as determined by MTT assay, was observed (Campisi et al., 2010). On the contrary, some studies did not detect such an association between MW irradiation and ROS levels (Lantow et al., 2006; Brescia et al., 2009). Lantow et al. investigated the effect of MW irradiation at 1800 MHz on ROS production and expression of heat-shock proteins (HSP70) in two human cell lines (Mono Mac 6 and K562). The authors did not detect a significant difference in ROS production or HSP70 expression as a result of microwave irradiation (Lantow et al., 2006).

1.6. Summary of literature work on the influence of microwave irradiation on biomolecules and biological systems

As mentioned above, the literature is divided on the existence of microwave-specific effects on biological systems (Banik et al., 2003). Difficulties in reproducing the results of some of the earlier studies on the specific impacts of MW irradiation has reduced confidence over the reliability of such data (Belyaev, 2005), although the conditions used in the repeated studies were not necessarily exactly the same as the original studies (Belyaev, 2005). It has to be acknowledged that considerable number of studies in which the bulk temperature of the system of study under microwave irradiation was kept relatively constant are also available (some of which are discussed in this review e.g. Vesper et al., 2005; Pavelkic et al., 2009; Asano et al., 2017; Nguyen et al. 2017). In this section, some of the recent studies on the biological effects of MW irradiation will be reviewed.

1.6.1. The effect of microwave irradiation on enzymatic activity

Many studies did not detect a significant impact on the enzymatic activity of the system investigated as a result of simultaneous or pre-treatment by microwave irradiation, at various frequencies and power intensities, of enzyme solutions and reactants (Galvin et al., 1981; Banik et al., 2003; Rejasse et al., 2007) Some reported different effects on the studied enzymes including deactivation or enhanced activity under MW irradiation (La Cara et al., 1999; Izquierdo et al., 2008; Chen et al., 2011). It is noteworthy that some

reports neglected the potential thermal impacts of the MW irradiation used, paid little to no attention in controlling the temperature of the system of study, or used unreliable methods to monitor the temperature, which could account for the observed effects.

Young et al. reported significantly enhanced activity of a β-glucosidase (CelB) isolated from *Pyrococcus furiosus*, which is a hyperthermophilic prokaryote of the domain Archaea, while under microwave irradiation (2.45 GHz, 300 W) at the bulk temperature range of -20 to 40 °C, which is far below the optimal temperature (110 °C) of the enzyme (Young et al., 2008). Similar enhanced activity of two other hyperthemophilic enzymes was observed when authors tested an α-galactosidase from *Thermotoga maritima* (Tm GalA) and a carboxylesterase from *Sulfolobus solfataricus* P1 (SsoP1 CE) under the same temperature and microwave irradiation profile (Young et al., 2008). The observed microwave-specific impact was dependent on the microwave power level, such that the lower the microwave power was, the less enhancement of enzymatic activity was observed compared to the control condition (0 W). However, the authors did not detect a significant impact on the activity of a mesophilic homologue of CelB isolated from *Prunus dulcisa* under the same conditions. No denaturation of any of the enzymes used in the study was observed after microwave irradiation, as determined by circular dichroism (CD) spectroscopy.

Pavelkic et al. reported decreased activity of porcine pepsin as a result of microwave irradiation (2.45 GHz, 30 W), while the bulk temperature was kept relatively constant (37 °C ± 1). The authors suggested that the observed effect could be due to microwave specific heating leading to increased autodigestion of pepsin under microwave irradiation

(Pavelkic et al., 2009). The results from SDS-PAGE (2D gel electrophoresis) showed increased amounts of smaller peptide fragments for the microwave irradiated enzyme solution compared to the control (non-irradiated), supporting the argument made by the authors in the aforementioned study (Pavelkic et al., 2009).

Some studies suggest that the impact of MW irradiation on enzyme activity is frequency dependent in addition to being power (intensity) dependent. Vojisavljevic et al. investigated the effect of MW irradiation at various frequencies within 500 to 900 MHz range on the enzymatic activity of L-Lactate Dehydrogenase (LDH). Thus, enzyme solutions were MW irradiated at very low powers (0.01, 0.1 and 1 μW) for 5 minutes prior to each reaction. The authors observed increased activity of LDH pretreated under MW irradiation at 500 MHz (0.01, 0.1 and 1 μW) and 900 MHz (only 1 μW) compared to the non-irradiated enzyme. MW irradiation at 650, 700 and 750 MHz (0.01, 0.1 and 1 μW) did not induce a similar impact (Vojisavljevic et al., 2011). All experiments were performed at room temperature (27 °C). However, the authors did not seem to monitor the temperature of the enzyme solution during the irradiation period nor any thermoregulatory mechanisms were utilized which could raise concerns over the reliability of data.

One of the recent applications of MW irradiation has been reported in proteomics. The method requires the enzymatic digestion of proteins prior to mass spectrometry which is often a lengthy process. Several studies report that MW irradiation could accelerate the enzymatic digestion of proteins (Vesper et al., 2005; Pramanik et al., 2009; Chen et al., 2011). Vesper et al. observed that the digestion efficiency of trypsin under MW

irradiation for 20 minutes and at 50 °C was higher than what could be achieved over 18 hours by conventional (through conduction) heating methods at the same temperature. The temperature of the sample during MW irradiation was monitored using a fiber optic probe with an accuracy of ± 2 °C. Under similar conditions, no significant improvement in the activity

of the enzyme glutamyl endopeptidase (Glu-C) under MW irradiation was observed. The authors suggested that this could be due to the differences in the stability of the two enzymes at the temperatures used, with trypsin having better stability and less likely to undergo heat inactivation at elevated temperatures compared to Glu-C (Vesper et al., 2005).

1.6.2. The effect of microwave irradiation on cellular growth and viability

Various studies with contradictory results have been published on the impact of MW irradiation on the growth of microorganisms. Mishra et al. investigated the effect of short (3 min and 6 min) MW treatment (2.45 GHz, 90 W) on the growth of *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus acidophilus* over 24 hours and the activity of certain enzymes expressed by the organisms. They did not detect a significant impact of MW irradiation on the growth of any of the three bacteria, as determined by measuring the optical density of bacterial suspensions (Mishra et al., 2013). However, the authors observed a significant increase (+27.24%) in Glucose-6-phosphatase activity in *B. subtilis* following the 6-minute MW treatment, but the activity of β-galactosidase was not significantly altered. Dholiya and coworkers also did not detect a significant impact on the growth of *B. subtilis* that underwent a similar MW treatment (2.45 GHz, 90 W, 6

min). Dholiya et al., however, found a significant increase in the growth (over 24 hours) of *E. coli* (which is contradictory to the results reported by Mishra et al., although the increased growth of *E. coli* in the study by Dholiya et al. was not dramatic (+2.3%)), *Staphylococcus aureus* and *Aspergillus parasiticus* that underwent the aforementioned MW treatment (Dholiya et al., 2012). Kushwah et al. reported that MW treatment (2.45 GHz, 90 W, 2, 4 and 6 min) stimulated the growth of *Streptococcus mutans*, while the growth of *B. subtilis* (consistent with the previously discussed studies) was unaffected (Kushwah et al., 2013). Raval et al. used a similar MW pretreatment of *Chromobacterium violaceum, Serratia marcescens*, and *Staphylococcus aureus* to investigate its impact on the growth and pigment production by the three bacteria. They found that MW treatment (2.45 GHz, 90 W, 6 min) did not significantly alter the growth of *C. violaceum*, but had a stimulatory effect on the growth of *Staphylococcus aureus* (in agreement with the study by Dholiya et al.) and had inhibitory impact on the growth of *Serratia marcescens* (Raval et al., 2014). Shorter MW pretreatments (2 and 4 min) of *Serratia marcescens* did not decrease the growth rate of *Serratia marcescens* but still had stimulatory impact on the growth of *Staphylococcus aureus*. The results suggest that the duration of MW exposure can be a key determinant of the outcome and is species-specific. Raval and coworkers found that the MW pretreatment (2, 4 and 6 min) could increase pigment production by *Staphylococcus aureus* but reduced pigment production in *C. violaceum* and *Serratia marcescens* suggesting that the effect is species-specific. Banik et al. observed that MW irradiation at various frequencies within 13.5-36.5 GHz could stimulate the growth of *Methanosarcina barkeri* DSM 804 (Banik et al., 2006). Of the range tested, the authors

reported that MW irradiation at 31.5 GHz had the most stimulatory impact on the production of methane by the bacteria.

Some studies have reported that microwave irradiation can cause the death of microorganisms at temperatures lower than the thermal-point that would be lethal when using conventional (through conduction) heating methods (Kozempel et al., 1998; Banik et al., 2003). This might suggest that the effective temperature that the microorganisms experienced under microwave irradiation could be higher than the average bulk temperature. Higher absorption of MW irradiation by certain components in microorganisms compared to the rest of the medium and or lower dissipation of heat, potentially due to the presence of cell membranes, could be two possible factors involved in the observed phenomenon. There are, however, other hypotheses in the literature, such as the interaction of the electric field of MWs with polar parts of enzymes or the ions in solution, causing alteration in the rate of certain biochemical reactions, ultimately leading to the reduced viability of the cells (Banik et al. 2003; Rai et al, 2009; Kushwah et al., 2013).

Asano et al. investigated the effect of MW irradiation (2.45 GHz, up to 20 W) on the viability of several human cell lines, both normal and cancerous (Asano et al., 2017). The temperature of the samples was monitored using an infra-red temperature probe and was maintained at 37 °C during the irradiation period (up to 3 hours). Following the MW irradiation treatment, cells were incubated for 24, 48 and 72 hours in a CO_2 incubator. After the incubation, the viability of the cells was determined using water soluble tetrazolium salt WST-8 assay and was compared to the control group that were not

microwave irradiated. Out of the 8 cell lines tested, 7 showed decreased viability as a result of MW irradiation. The normal breast cell line MCF-12A was the only cell line that did not show reduced viability as a result of MW irradiation. Also, the cell lines that showed reduced viability were not equally sensitive to MW irradiation. For example, the viability of the gastric cancer cell line KATO III was reduced more drastically compared to the gastric non-cancer cell line HGC-27. The authors suggested that different cell lines have different susceptibility to MW irradiation. It was also shown that there is a negative correlation between the irradiation time and the viability of the cells as well as between the output power of MW irradiation and the viability of the cells. However, the usage of an infra-red temperature probe in the study, which was placed underneath the cell plates, instead of having a temperature probe inside the sample, that were being irradiated raises concerns over how accurately the measured temperature represents the actual temperature of the samples during MW irradiation. This uncertainty in the temperature measurement leaves open the possibility that the reduced viability observed in the study could be due to the overheating of the cells.

1.6.3. The effect of microwave irradiation on the cell membrane permeability and uptake of compounds by cells

Several studies have been reported on the impact of MW irradiation on the cell membrane permeability of microorganisms. Increased cell membrane permeability of several bacteria and yeast under MW irradiation has been reported over the past 15 years (Barnett et al., 2006; Shamis et al., 2011; Rougier et al., 2014; Zeng et al. 2014; Nguyen et al., 2015).

Nguyen and coworkers investigated the effect of MW irradiation at the frequency of 18 GHz (SAR: 5.0 kW/kg) on the bacterial membrane permeability of four Gram-positive bacteria (*Planococcus maritimus* KMM 3738, *Staphylococcus aureus* CIP 65.8T, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 14990T) and *E. coli* ATCC 15034, a Gram-negative bacterium. In the study, bacterial suspensions were MW irradiated three times at 18 GHz for 1 minute. The temperature of the cultures was allowed to increase up to 40 °C in the MW irradiation period which was followed by 2-minute cooling period to let the cultures cool down to 20 °C. The bulk temperature of the samples was measured using a fiber optic temperature probe. A propidium iodide solution (1 mg/ml) was added to the bacterial suspensions 1 and 10 minutes after the microwave

exposure. Using confocal laser scanning microscopy (CLSM), it was found that the bacterial cells of the five species tested could take up propidium iodide that was added 1 minute after the MW irradiation but not 10 minutes after the exposure (Nguyen et al., 2015). However, the control group of cells, which were subjected to the same temperature profile as the MW irradiated cells but using conventional (through conduction) heating methods, were unable to take up propidium iodide. The study also investigated the uptake of fluorescent silica nanospheres (23.5 and 46.3 nm in diameter) by bacteria under microwave irradiation. It was found that MW irradiation could induce the uptake of 23.5 nm nanospheres by all five bacteria, but not all could take up the 46.3 nm nanospheres. The uptake of nanospheres by the control group was far below the level of MW-treated cells (Nguyen et al., 2015).

Another study by Nguyen et al. investigated the effect of high frequency (18 GHz, SAR: 3.0 kW/kg) microwave irradiation on the uptake of two types of fluorescent silica nanospheres (23.5 and 46.3 nm in diameter) by red blood cells. In the study, temperature of the samples was monitored using a fiber-optic probe and was kept under 37 °C during the irradiation period to prevent over-heating of the cells. The uptake of the nanospheres by red blood cells was investigated using CLSM, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The results obtained from the three methods indicated that the uptake of the nanospheres was significantly enhanced under MW irradiation compared to the control (no irradiation at the same bulk temperature), where no uptake of the nanospheres by red blood cells was observed under the control condition. SEM results also indicated that the original morphology of the red blood cells was preserved after MW irradiation. However, the authors could not rule out the possibility of local heating under MW irradiation, leading to localized temperature elevation and increased cell membrane permeability, as a potential cause for the observed phenomenon (Nguyen et al., 2017).

1.6.4. Conclusion

Based on the results reported in the literature, MW irradiation could induce effects on the biological systems at different levels which were significantly different from what was observed with conventional (through conduction) heating methods. The results suggest that the impact of MW irradiation can be dependent on various factors including the frequency, intensity and duration of exposure. The results also suggest that the MW specific effect can be enzyme or species specific. Selective heating or local hot spot

formation, however, could still be thought of as the potential causes of some of the observed effects that could not be ruled out by majority of the studies reported, although it might be difficult to account for some of the reported effects (e.g. activation of VGCCs) solely based on the thermal properties of MW irradiation. Application of systems that would monitor the temperature profile of the whole components of samples (specially at microscopic scale) under MW irradiation in real time might help to elucidate some of these uncertainties. For instance, if selective heating is responsible for the observed increased cell membrane permeability under MW irradiation, it would be crucial to find out which parts or components of cell membranes are absorbing MW irradiation more efficiently and how much warmer those regions are compared to the rest of the cell.

Work described in this thesis investigated the influence of microwave irradiation on activities of selected enzymes, cell membrane properties, and bacterial growth. In this work, a CEM microwave equipped with a Coolmate was used as it allows for the cooling of systems of interest while subjected to microwave irradiation. This microwave system operates at 2.45 GHz, and the power output is adjustable from 5–300 W; however, the power output did not exceed 10 W for all the work (except for chapter 2 in which the power output did not exceed 20 W) described in this thesis in order to minimize the issue with overheating. Furthermore, the temperature of systems studied in this thesis was regulated usually ±1 °C within the set point, through simultaneous cooling, with temperature monitored via a fibre optic temperature probe directly immersed into the system under investigation.

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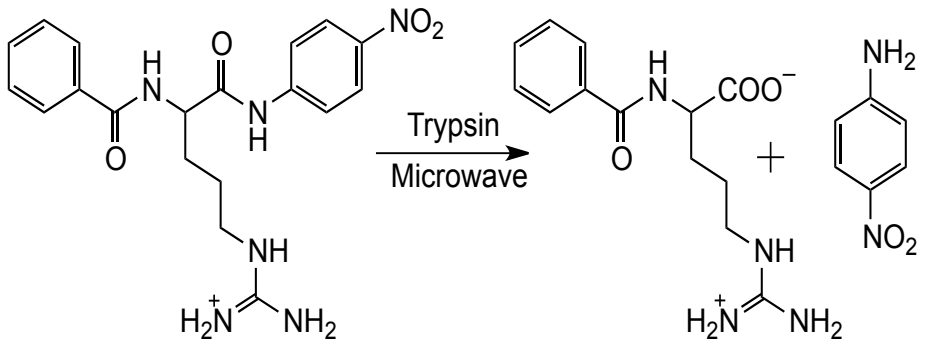
Chapter 2

Microwave radiation accelerates trypsin catalyzed peptide hydrolysis at constant bulk temperature

Microwave heating has become an increasingly popular heating method in a wide range of applications over the past few decades. In organic synthesis, microwave heating has been shown to be very useful, often leading to faster reactions, higher yields, and better selectivity.[1-6] One of the areas that has attracted heated debate relates to whether "non-thermal" or "microwave specific effects" exist.[7-12] We are interested in the effect of microwave irradiation on reactions that involve biomolecules such as enzymes, particularly when the reaction mixture is maintained at constant bulk temperature while exposed to low power microwave (less than 20 W, SAR: less than 1400 mW/ml), as these experiments could provide some insight into the influence of microwave exposure on biological systems, where the macroscopic temperature is mediated by the bulk surroundings.

While some work has demonstrated that microwave radiation affects protein/enzyme structures,[13-16], bulk literature evidence has suggested that enzymatic activity can be affected when enzymes are exposed to microwave. The literature in this area prior to 2007 is summarized in a review.[17] This area of research was further demonstrated in more recent work.[18-30] It is worthwhile noting that among the challenges in establishing reproducible results in these observations, monitoring and regulation of reaction temperature accurately has been the most difficult one.[31] In this respect, Kappe and co-workers[32] showed that there was no difference in reactivity and enantioselectivity in the kinetic resolution of *rac*-1-phenylethanol catalyzed by immobilized lipases under conventional (through conduction) or microwave heating, when the temperatures were maintained the same. In this work, we further explored the influence of microwave exposure on trypsin activity while the bulk reaction temperature is kept constant through external cooling.

Towards this goal, trypsin from the bovine pancreas was chosen as the enzyme and a dipeptide, *N*α(±)-benzoyl-D/L-arginine 4-nitroanilide hydrochloride (BAPNA), as the substrate (Scheme 1). BAPNA is readily hydrolyzed by trypsin to give *N*-benzoyl arginine and *p*-nitroaniline. As the latter can be quantified readily by a colorimetric assay, this system provides an easy approach to study the effect of microwave irradiation on enzyme activity.



Scheme 2.1. Hydrolysis of *N*α(±)-benzoyl-D/L-arginine 4-nitroanilide hydrochloride by trypsin.

In order to maintain reaction temperature while the reaction mixture is exposed to microwave, the CEM CoolMate Microwave System (Figure 2.1) was used. This microwave reactor operates at a frequency of 2.45 GHz, and provides the ability to keep constant bulk reaction temperatures while the reaction mixture is exposed to microwave irradiation. This system features a reaction vessel circumvented by a jacket where a microwave transparent (substances that have very low specific absorption rate under microwave irradiation) fluid, such as perfluoropolyether Galden HT 110, circulates. The coolant is pre-cooled in a reservoir by either dry ice or liquid nitrogen. An important feature of this setup is that the reaction temperature is monitored *in situ* with an optic

fiber temperature probe, which provides feedbacks to the system so that the microwave output can be adjusted in a real-time fashion to maintain a pre-determined temperature in the reaction mixture.

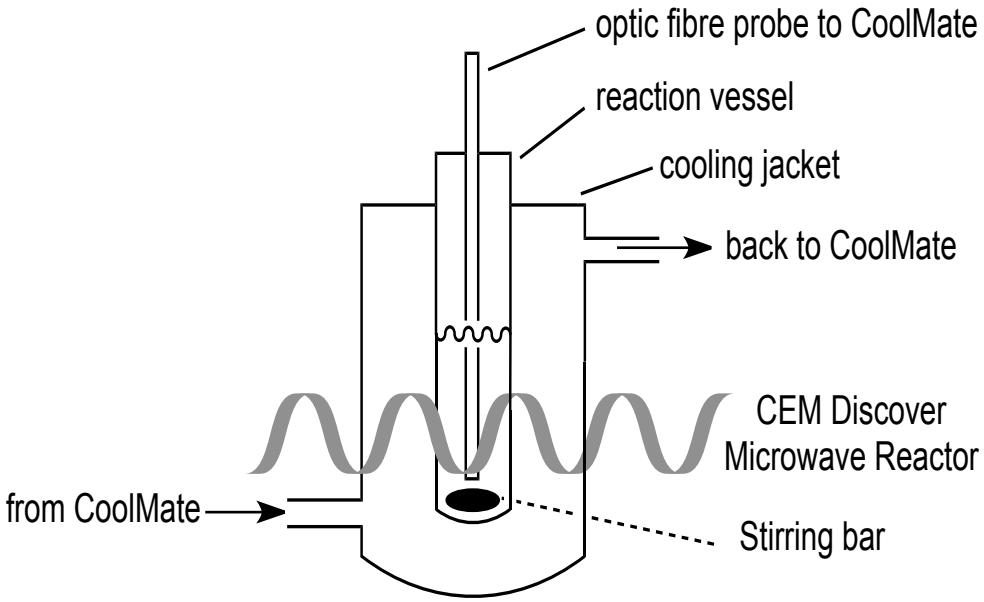


Figure 2.1. The CEM CoolMate Microwave System that is used in this study.

When BAPNA was subjected to trypsin digestion, it became clear that hydrolysis was accelerated by microwave radiation, while the bulk reaction temperature was kept constant by simultaneous cooling. Figure 2.2 shows the progress of two reactions carried out at 25°C, as measured in the absorbance of product formed over time, one in the absence of microwave (control), and another exposed to up to 20 W microwave radiation while the temperature was maintained at 25°C. Indeed, the increase in the rate of reaction through microwave radiation was observed when the reactions were carried out under other

conditions, such as 37 °C and various trypsin concentrations (data not shown); however, in order to derive a quantitative comparison of enzyme properties, it became clear that BAPNA is not a suitable substrate due to its limited solubility and, as a consequence, low absorbance of the product formed.

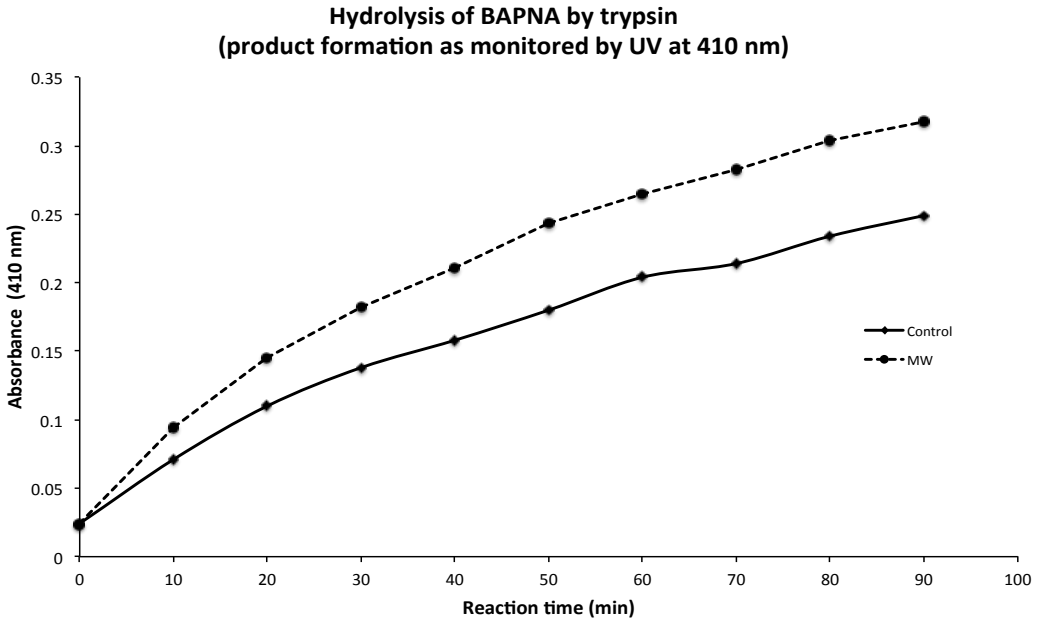


Figure 2.2. Digestion of BAPNA (1.0 mM) by trypsin (5.0 μM). Solid line: reaction at 25 °C in the absence of microwave; dashed line: reaction mixture was exposed to microwave radiation of varying power of up to 20 W, while the bulk temperature was kept at 25 °C through external cooling by a CoolMate system.

We next examined the effect of microwave radiation on the rate of digestion of azocasein by trypsin.[33] Similar trends, that is, acceleration of the rate of hydrolysis by microwave, were observed. Despite the challenges in generating a standard curve that correlates absorbance with concentration of product formed, which led to difficulty in obtaining quantitative comparison, a plot of changes in absorbance at 440 nm of samples taken from the reaction mixture five minutes after the reaction started at a range of initial azocasein concentrations (Figure 2.3) clearly indicated an increase in the reaction rate, specially at

higher initial azocasein concentrations. The results suggest that while trypsin is presumably saturated at low concentrations of azocasein, at higher azocasein concentrations, digestion of the substrate by trypsin is accelerated by microwave irradiation while the bulk temperature is kept the same as in the control.

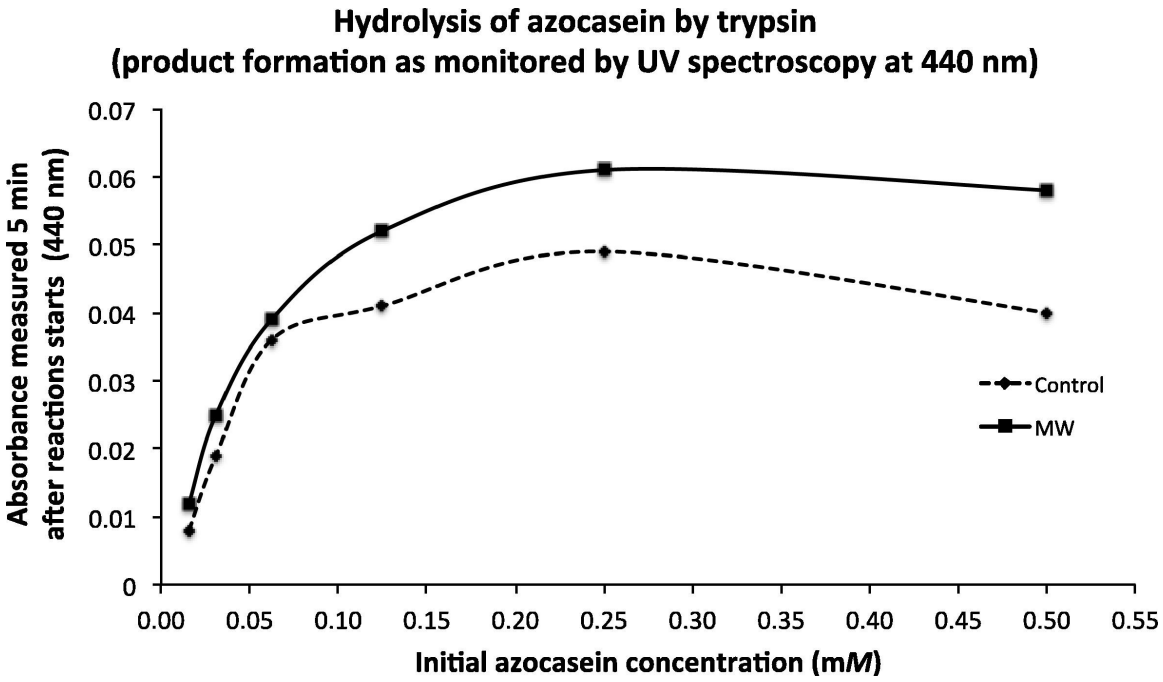


Figure 2.3. Plot of changes in absorbance at 440 nm of samples taken 5 minutes after reactions initiate, versus initial azocasein concentrations. Both control (reactions carried out in the absence of microwave) and microwave (exposed to microwave radiation of varying power of up to 20 W) reactions were carried out at 22°C.

In order to derive a quantitative comparison of trypsin kinetics, casein was subsequently used as trypsin substrate as a calibration curve can be readily determined.[34] Due to the constraint on the volume of the microwave reaction vessel, K_m and V_{max} values were not determined; instead, the trypsin activity (or 'apparent' activity in the presence of microwave) was determined at a range of casein concentrations.

As is shown in Figure 2.4, compared with untreated control, the trypsin activity is increased when it was exposed to microwave irradiation while the bulk temperature was maintained constant at 22 °C (±2 °C). The increased activity is particularly profound at high casein concentrations where trypsin is fully saturated. Note that in these microwave experiments, the reaction mixtures were exposed to 10 W (9-10 W) microwave, while the temperature was controlled at 22 °C (±2 °C), by a constant coolant flow. In addition to the temperature measurement by an optic fibre temperature probe, the bulk temperatures of the reaction mixtures were also confirmed by inserting an alcohol-based thermometer directly into the solution.

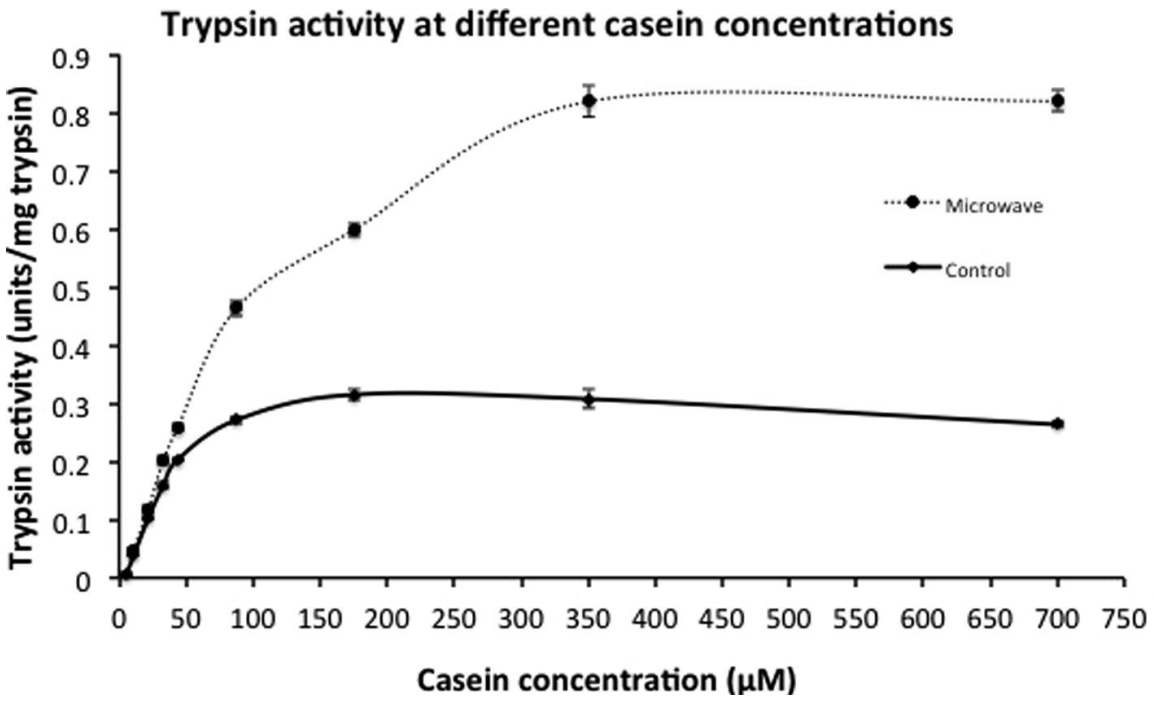


Figure 2.4. Trypsin activity determined at different casein concentrations. Data are triplicate for both control and microwave experiments. Microwave reactions were carried out with exposure to 9-10 W microwave while the temperature was maintained at 22 °C (±2°C). Definition of a unit: the amount in micromoles of L-tyrosine equivalents released from casein per minute. Flow rates of the CoolMate system were manually adjusted so that a constant bulk temperature can be maintained. Error bars are shown as standard deviation.

In order to demonstrate the cooling effected by the CoolMate system, a control experiment was carried out where the casein reaction mixture was exposed to a constant 10 W microwave heating, however, without simultaneous cooling by the CoolMate system. It is quite clear from Figure 2.3S in the Supplementary Material that the temperature as measured by a fiber optic probe rose from 18 °C to 40 °C in approximately 4 min.

Circular dichroism profiles (Figure 2.5) of freshly prepared trypsin solution, trypsin solution exposed to up to 20 W microwave radiation at 37 °C and conventional heating

(through conduction) at 37 °C suggest that changes to trypsin structure occur when trypsin is either heated or exposed to microwave. These changes are most probably attributed to self-catalyzed cleavages that occur over time.[35]

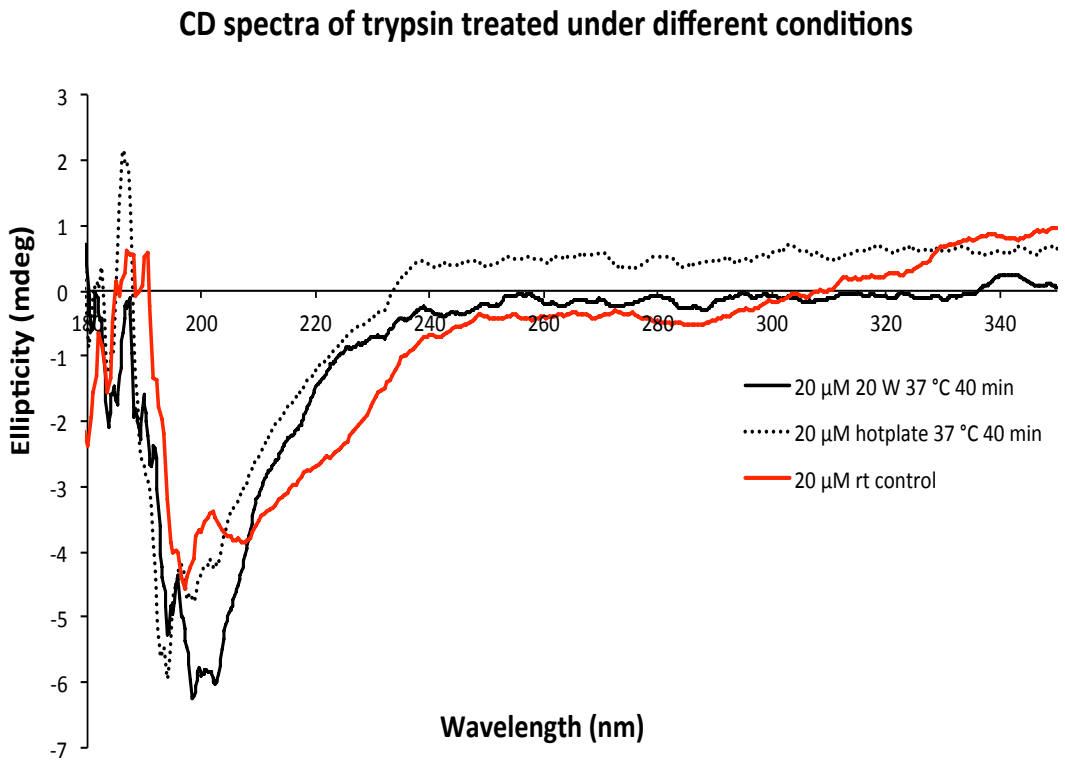


Figure 2.5. CD spectra of trypsin: freshly prepared and untreated (red solid line), heated at 37 °C in an oil bath (black dashed line) for 40 minutes, and heated by up to 20 W microwave for 40 minutes while the bulk temperature was maintained at 37 °C (black solid line).

To summarize, hydrolysis of substrates catalyzed by trypsin is accelerated by microwave irradiation while the bulk temperature of the reaction mixture is kept constant through

external cooling. It is possible that this rate acceleration is caused by the formation of local hot spots during microwave irradiation, presumably as a consequence of selective heating as has been suggested in recent literature.[36]

Acknowledgments

This research was supported by the Natural Science and Engineering Research Council of Canada. The authors also wish to thank Dr. Jean-Paul Desaulniers for assistance with circular dichroism measurement.

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Supplementary Material

Experimental procedures for this work can be found in the Supplementary Material.

Materials and methods

Supplementary Material

Microwave radiation accelerates trypsin-catalyzed peptide hydrolysis

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Note: All experiments were performed by Sina Atrin Mazinani.

Experimental

All chemicals and reagents were purchased from Sigmaaldrich without further purification. MilliQ water was used for all experiments. A CEM Discovery Coolmate microwave system was operated at 2.45 GHz. Fiber optic Model: CEM Discover, fiberoptic (Part Number 314307, range -50 °C to 250 °C, Made in Canada). Temperature reads of the fiber optic probe was compared to the reads of alcohol-based thermometers regularly to ensure their accuracy. Colorimetric assay of the trypsin digestion was performed with a Biotek Microtitre Plate Reader using a Gen 50 software. Circular Dichroism measurement was carried out with a Jasco J-815 CD Spectrophotometer. All stock solutions were prepared fresh on the day of the experiment. Replicates of each experiment were not necessarily conducted on the same day. To ensure thermal homogeneity a stirring bar (1×8 mm) was used at 800 rpm. Power density at 10 W is 700 mW/mL. The potential effect of the stirrer on the EMF created by MW irradiation is not known.

Trypsin digestion of *N*α(±)-benzoyl-DL-arginine 4-nitroanilide hydrochloride

Trypsin stock solution (20 μM) and BAPNA stock solution (1 mM) were freshly prepared prior to reactions. For each digestion experiment, the trypsin stock solution (296 μl) was added to a solution of BAPNA (740 μl), 2200 μl Tris-HCl (40 mM, pH 7.7) and 740 μl $CaCl_2$ (10 mM). Aliquots (270 μl) were withdrawn from the reaction mixture every 10 minutes, and added to a 96-well quartz plate. Absorbance was measured at 410 nm using a Biotek Powerwave xs plate reader. Initial absorbance was measured at time=0.

Control experiments were carried out in the microwave reaction vessel in a water bath on a hotplate without microwave irradiation at 25°C. Using the same reaction vessel is important as different reaction vessels/containers could lead to varying degrees of adsorption of trypsin on the surface, and thus change the reaction kinetics.

Microwave experiments were carried out using a CEM Coolmate reactor. Temperature of the reaction mixture was kept constant (at 25°C), as monitored by an optic fibre temperature probe that was inserted into the reaction mixture, by flowing coolant through the cooling jacket around the reaction vessel. In these experiments, the maximal microwave power was set at 20 W, however, the actual microwave power was adjusted by the Synergy software that controls the microwave reactor, based on the temperature feedback from the optic fibre temperature probe.

Digestion of azocasein by trypsin

Trypsin stock solution (10 μM in 10 mM calcium chloride) was freshly prepared prior to reactions. For each digestion experiment of azocasein, the trypsin stock solution (1.0 ml) was added to varying dilutions (1.0 ml) (Table 2.1S) of azocasein stock solution (2 mM in 0.5% $NaHCO_3$, pH 7.8). After 5 minutes, trichloroacetic acid (5% aqueous solution, 8.0 ml) was added to terminate the reaction. Aliquots (270 μl) were taken and added to a 96-well quartz plate. Absorbance was measured at 440 nm using a Biotek Powerwave xs plate reader.

Table 2.1S. Concentrations of azocasein dilutions used in the digestion experiments.

Azocasein concentration (mM)	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625

Control experiments were carried out in the microwave reaction vessel in a water bath on a hotplate stirrer without microwave irradiation at 22°C. Using the same reaction vessel is important as different reaction vessels/containers could lead to varying degrees of adsorption of trypsin on the surface.

Microwave experiments were carried out using a CEM Coolmate reactor. The temperature of the reaction mixture was kept constant at 22°C, as monitored by an optic fibre temperature probe that was inserted into the reaction mixture, by running coolant through the cooling jacket around the reaction vessel. In these experiments, the maximal microwave power was set at 20 W, however, the actual microwave power was adjusted by the Synergy software that controls the microwave reactor, based on the temperature feedback from the optic fibre temperature probe.

Digestion of casein by trypsin

Trypsin stock solution (20 μM in 10 mM sodium acetate and 5 mM calcium chloride) was freshly prepared prior to reactions. Casein stock solution (2% in 50 mM potassium phosphate dibasic) was prepared with pH adjusted to 7.5. For each digestion experiment, the trypsin stock solution (1.0 ml) was added to varying dilutions of casein solution (5.0 ml) (Table 2.2S). After 10 minutes, trichloroacetic acid (5% aqueous solution, 5.0 ml) was added to terminate the reaction. The mixture was incubated for 30 minutes at 22 °C and was then filtered through a 0.45 μm syringe filter. To the filtrate (only 2.0 ml of the filtrate was used in the following steps) were added of sodium carbonate (500 mM, 5.0 ml) and Folin's Phenol Reagent (0.5 M, 1.0 ml). The mixture was incubated at 22 °C for 30 minutes and then filtered through a 0.45 μm syringe filter. Aliquots (270 μl) were removed and added to a 96-well quartz plate. Absorbance was read at 660 nm using a Biotek Powerwave xs plate reader.

Table 2.2S. Concentrations of casein dilutions used in experiments.

Casein concentration (g/100ml)	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625

Control experiments were carried out in the microwave reaction vessel without microwave irradiation at 22°C. Using the same reaction vessel is important as different reaction vessels/containers could lead to varying degrees of adsorption of trypsin on the surface. Enzyme was added at the beginning of the experiment.

Microwave experiments were carried out using a CEM Coolmate reactor. The temperature of the reaction mixture was kept at 22 °C (±2 °C)., as monitored by an optic fibre temperature probe that was inserted into the reaction mixture, by running coolant through the cooling jacket around the reaction vessel. In these experiments, microwave power was set at 10 W, without going lower than 9 W throughout the reaction, while the temperature of the reaction mixtures was maintained at 22 °C (±2 °C) through cooling.

Generation of L-tyrosine standard curve

L-Tyrosine stock solution (1.1 mM) was prepared. To various dilutions of L-tyrosine (2.0 ml, see Table 2.3S for dilutions) were added sodium carbonate (5.0 ml, 500 mM) and Folin's Phenol Reagent (1.0 ml, 0.5 M). The reaction mixture was incubated at 22 °C for 30 minutes and then filtered through a 0.45 µm filter. Aliquots (270 µl) were taken and added to a 96-well quartz plate. Absorbance was measured at 660 nm using a Biotek powerwave xs plate reader. The standard curve was created by plotting the absorbance reads as a function of L-tyrosine concentration (Figure 2.1S).

Table 2.3S. Concentrations of L-tyrosine dilutions.

L-tyrosine concentration (mM)	0.275	0.220	0.110	0.055	0.0275

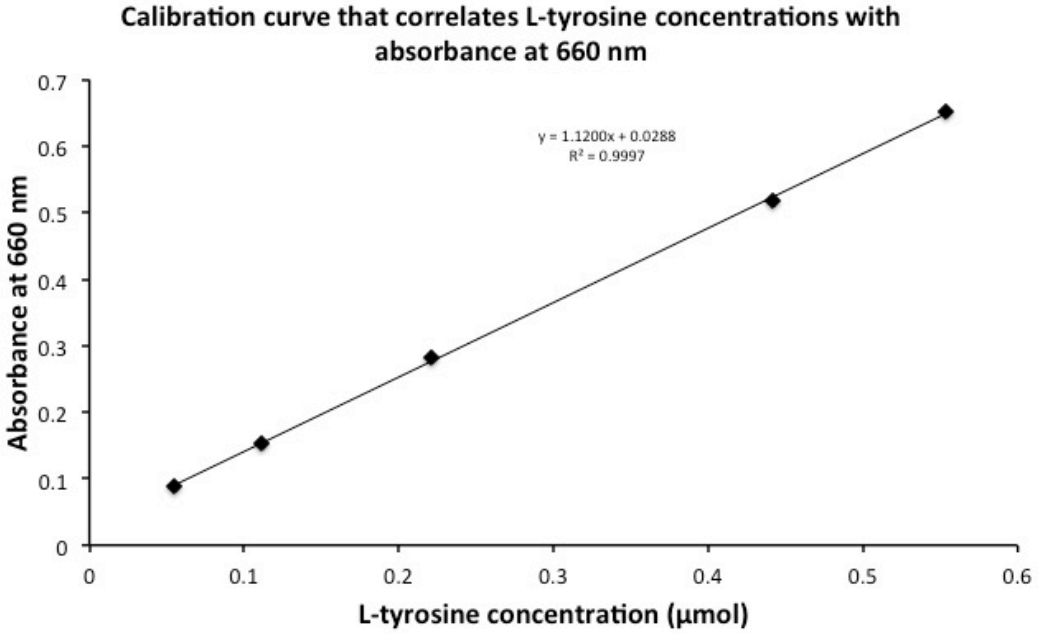

Figure 2.1S. Calibration curve that correlates L-tyrosine concentrations with absorbance at 660 nm in the casein digestion assay.

Calculation of trypsin activity

Calculation of trypsin activity was based on a literature procedure (Cupp-Enyard, C. *J. Vis. Exp*. **2008**, *19*, e899), which is also documented by a Sigmaaldrich product protocol (Universal Protease Activity Assay: Casein as a Substrate - See more at: http://www.sigmaaldrich.com/life-science/learning-center/life-science-video/universal-protease.html#sthash.K2BQQ5Nq.dpuf).

Measurement of CD spectra of trypsin

CD scans were performed in a cuvette with 1 mm path length at 25°C. Samples were scanned at 170–350 nm at a speed of 100 nm/min, with 1.0 nm increments. Sensitivity of the scans was set at high. A total of four scans were accumulated for each sample.

Trypsin solutions (0.4 mM in 40 mM Tris-HCl buffer, pH 8.0) were (a) untreated, *i.e.* left to stand at room temperature, (b) heated at 37 °C in an oil bath, or (c) exposed to up to 20 W microwave irradiation while the bulk temperature was maintained at 37°C. After 40 minutes, samples (400 µl) were withdrawn for CD measurement.

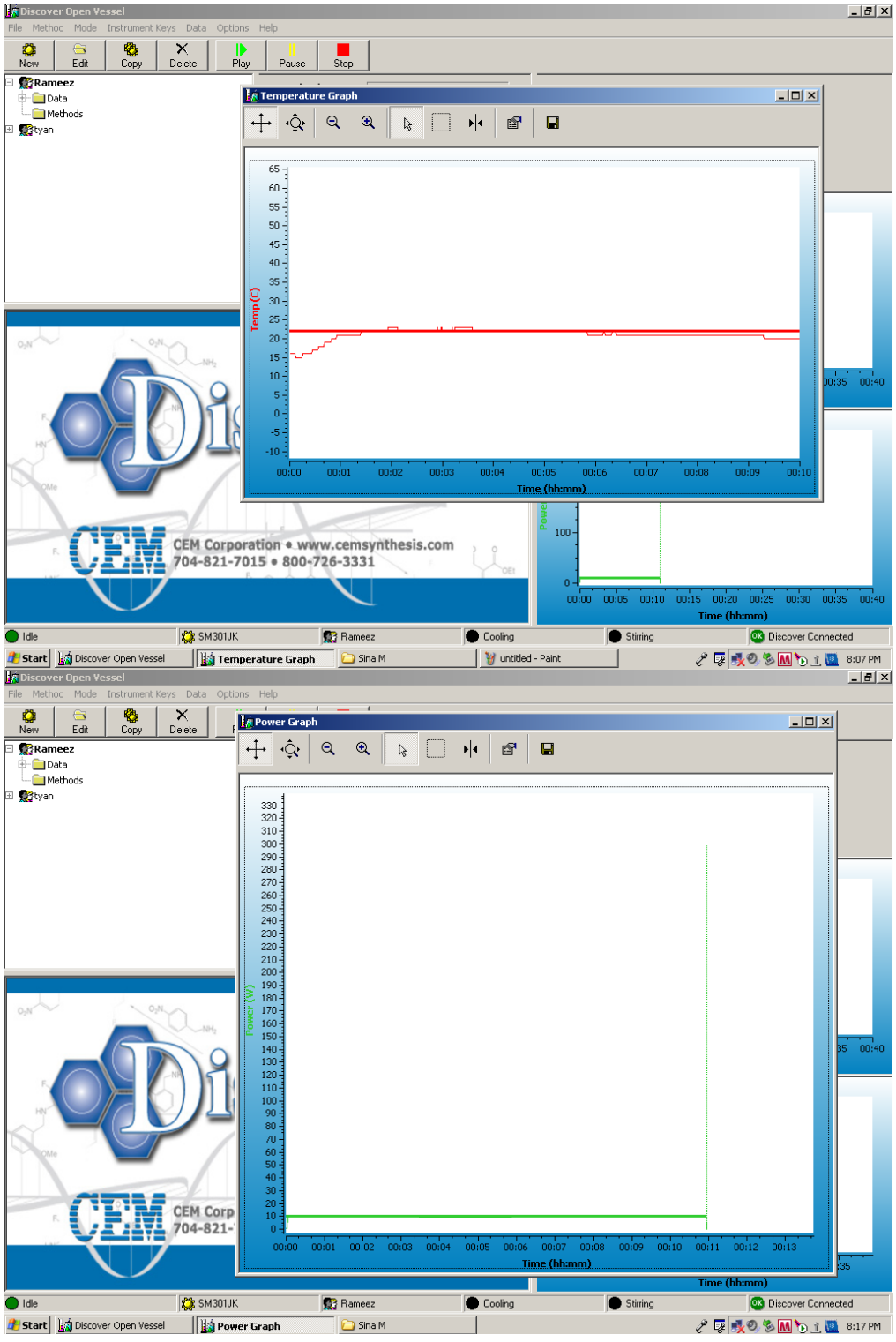

Figure 2.2S. Representative screenshot of the temperature/microwave power profiles for the casein experiment (top: temperature; bottom: microwave power). Temperature monitored by an optic fibre temperature probe *in situ*. Power was kept within 9-10 W.

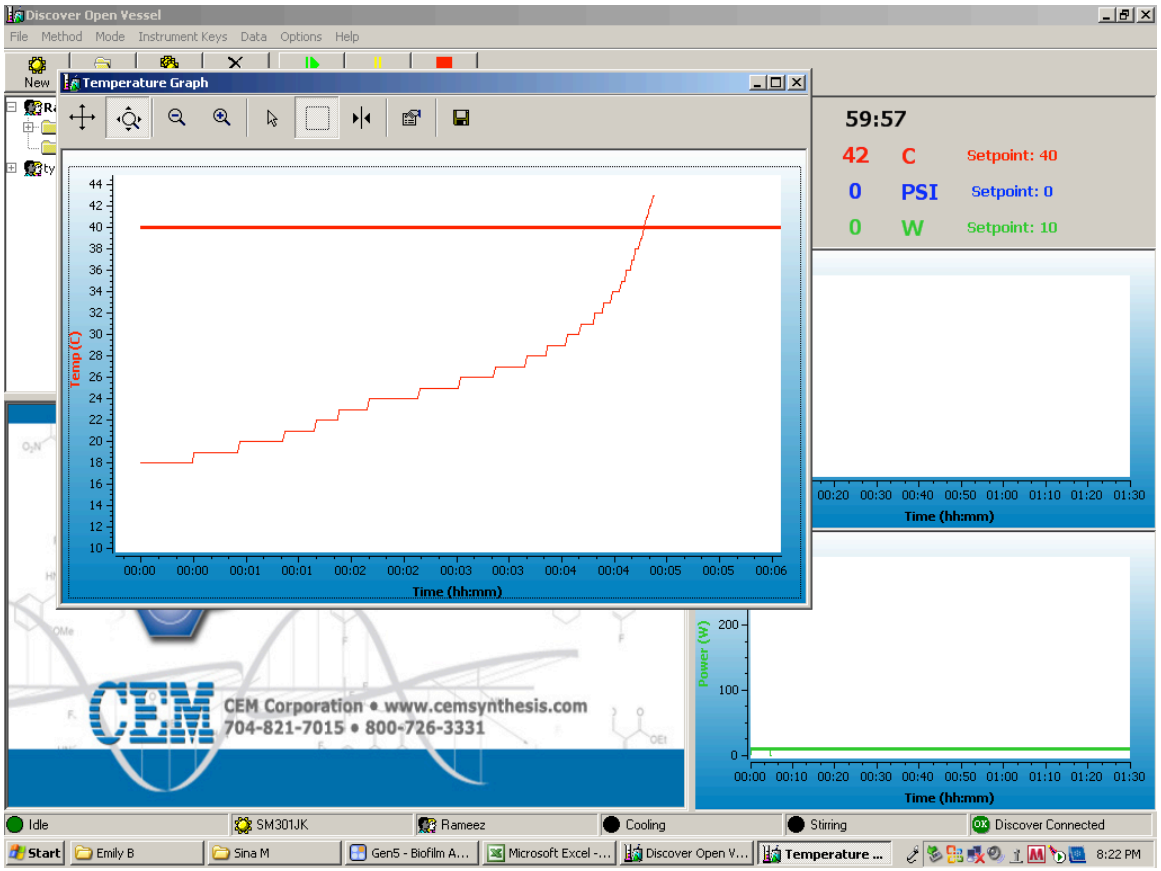

Figure 2.3S. Temperature profile of the casein reaction mixture exposed to constant 10 W microwave, while no cooling was provided by the CoolMate. The temperature of the mixture rose from 18 °C to 40 °C in approximately four minutes.

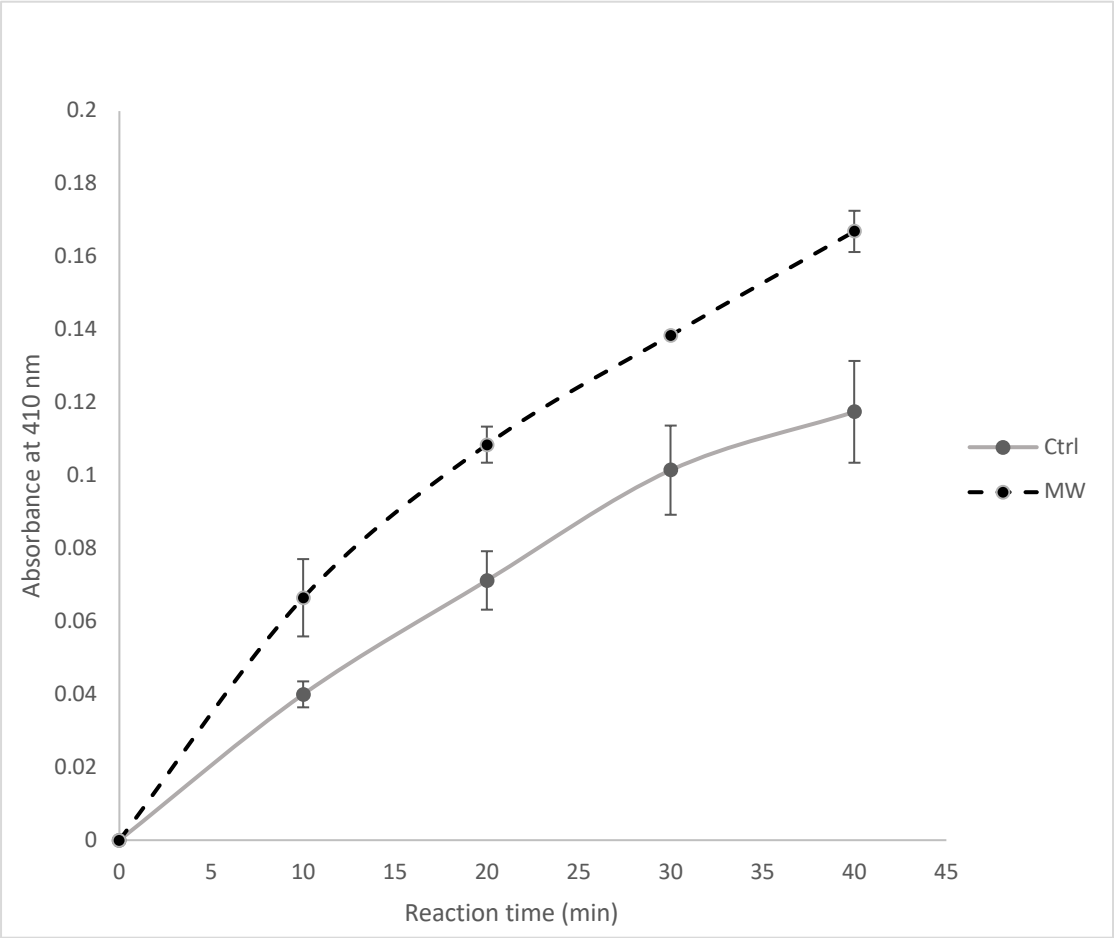

Figure 2.4S. Digestion of BAPNA (1.0 mM) by trypsin (5.0 μM). Solid line: reaction at 25 °C in the absence of microwave; dashed line: reaction mixture was exposed to microwave radiation of varying power of up to 20 W, while the bulk temperature was kept at 25 °C through external cooling by a CoolMate system (Control n=5, MW n=2). Initial absorbance was blanked in each experiment. Error bars are shown as standard deviation.

Chapter 3

Increase of enzymatic activity by microwave irradiation at constant bulk temperature is enzyme-dependent

In recent years, microwave heating has attracted increasing interest as an effective method to increase rates of organic reactions, and in some cases improve reaction outcomes.[1-4] This heating method has also been successfully used in solid phase peptide synthesis[5] and material chemistry.[6-8] One important aspect of microwave heating that has raised significant debate is the origins of the different outcomes of reactions effected by microwave or conventional heating (through conduction). Towards these discussions, the "microwave-specific effect" has been frequently referred to in the literature[9-14] as a consequence of "selective heating" of certain reactants in the presence of microwave.[15,16]

While the literature divides in the influence of microwave on reactions when the reaction temperature is kept constant, we recently demonstrated that trypsin activity is significantly increased upon exposure to relatively low power microwave (not more than 20 W), and while the bulk temperature of the reaction mixture is kept constant through simultaneous cooling.[17] An important consideration towards a better understanding of the "microwave-specific effect" is to also examine the effect of microwave on other enzymes at constant bulk reaction temperature. Towards this objective, we here report the impact of relatively low power microwave (10 W, SAR: 700 mW/ml) on the activity of α-amylase and phosphatase, while the reaction temperature is maintained virtually constant.

First, 4-nitrophenyl phosphate was subjected to hydrolysis by alkaline phosphatase from bovine intestinal mucosa. As a control experiment, the reaction was carried out in a CEM microwave reaction vessel at 22°C, however, not exposed to microwave. Another phosphatase-catalyzed hydrolysis reaction was carried out in a CEM Discover Reactor equipped with a CoolMate system. The latter reaction was subjected to 10 W constant microwave while the reaction temperature was maintained constant at 22 °C through

simultaneous cooling. In our previous report with trypsin, a fiber optic temperature probe and an alcohol-based glass thermometer were both used to follow the bulk reaction temperature, and both were found to give consistent values. In order to simplify the experimental set-up, an alcohol-based glass thermometer was used to monitor reaction temperature throughout the experiments with phosphatase and amylase. Aliquots of reaction solutions were removed after 30 minutes, and absorbance of each sample was determined at 405 nm.[18]

It is quite clear from Figure 3.1 that alkaline phosphatase activity is virtually unaffected by exposure to microwave while the bulk reaction temperature is maintained constant. As a control experiment, it is worthwhile noting that the reaction temperature rises from 18 °C to 40 °C in about 4 minutes, when simultaneous cooling is not provided (Figure 2.3S).

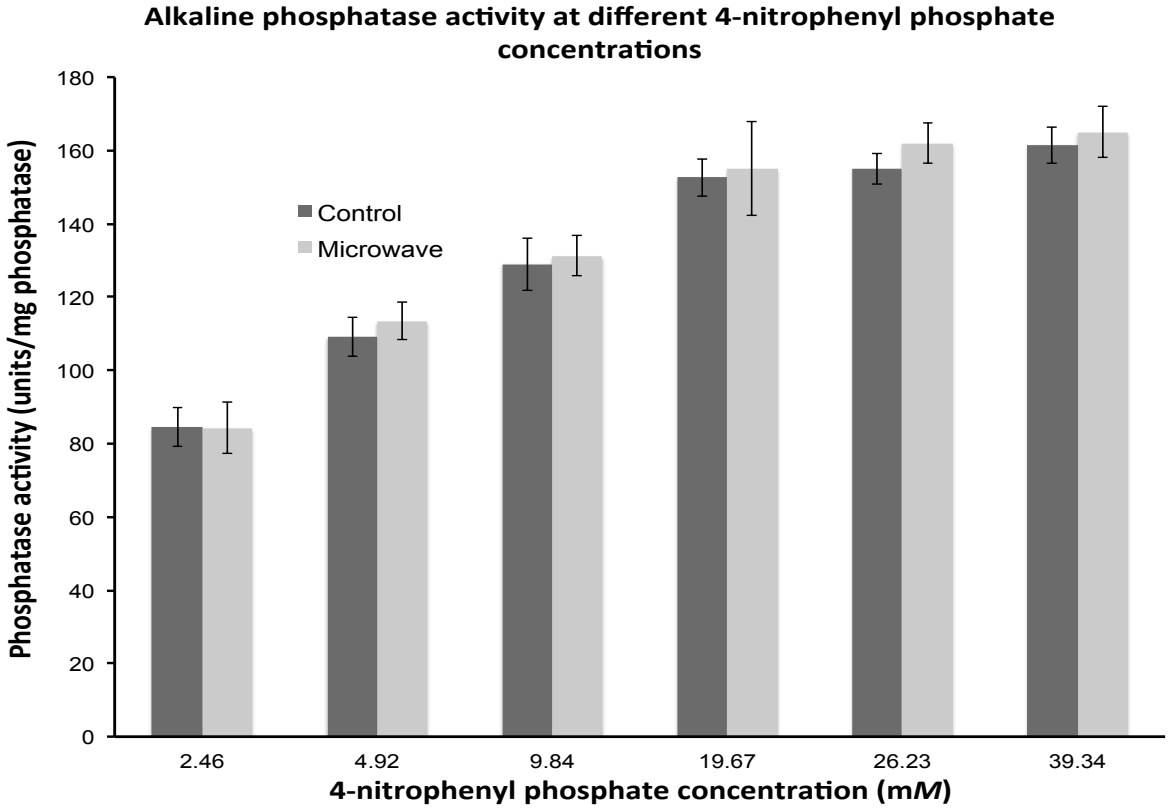

Figure 3.1. Comparison of alkaline phosphatase activity in digesting 4-nitrophenyl phosphate in the presence and absence (control) of 10 W constant microwave. Bulk temperatures of both experiments were maintained at 22°C. All values are average of triplicate experiments. Note that the flow rate of the CoolMate system in each experiment was manually adjusted to maintain constant bulk temperatures. One unit will hydrolyze 1.0 μmole of 4-nitrophenyl phosphate disodium salt hexahydrate per 30 minutes at pH 10.0 at 22°C. Error bars are shown as standard deviation.

Examination of the impact of 10 W microwave irradiation on the activity of α-amylase, while the bulk reaction temperature remains constant, revealed similar results (Figure 3.2). Thus, when the digestion of starch by α-amylase[19] was subjected to constant 10 W microwave irradiation, while cooled simultaneously to maintain the reaction temperature

at 23°C, there is virtually no difference in the activity of α-amylase, compared with those in the absence of microwave.

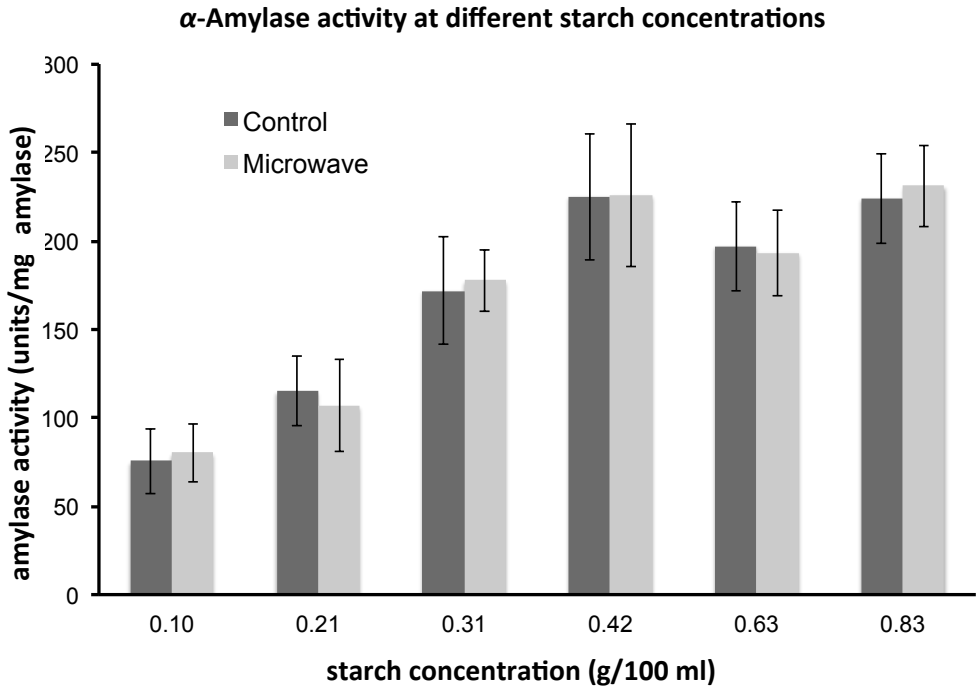

Figure 3.2. Comparison of α-amylase activity in digesting starch in the presence and absence (control) of 10 W constant microwave. Bulk temperatures of both experiments were maintained at 23°C. All values are average of triplicate experiments. Note that the flow rate of the CoolMate system in each experiment was manually adjusted to maintain constant bulk temperatures. One unit will liberate 1.0 mg of maltose from starch in 5 minutes at pH 6.9 at 23°C. Error bars are shown as standard deviation.

These results are in sharp contrast to those of trypsin experiments that we previously reported.[17] As can be seen from the data (Figure 3.3) reformatted from our previous studies, the trypsin activity towards the digestion of casein is increased very significantly by exposure to low power microwave (10 W) while the reaction temperature is maintained constant.

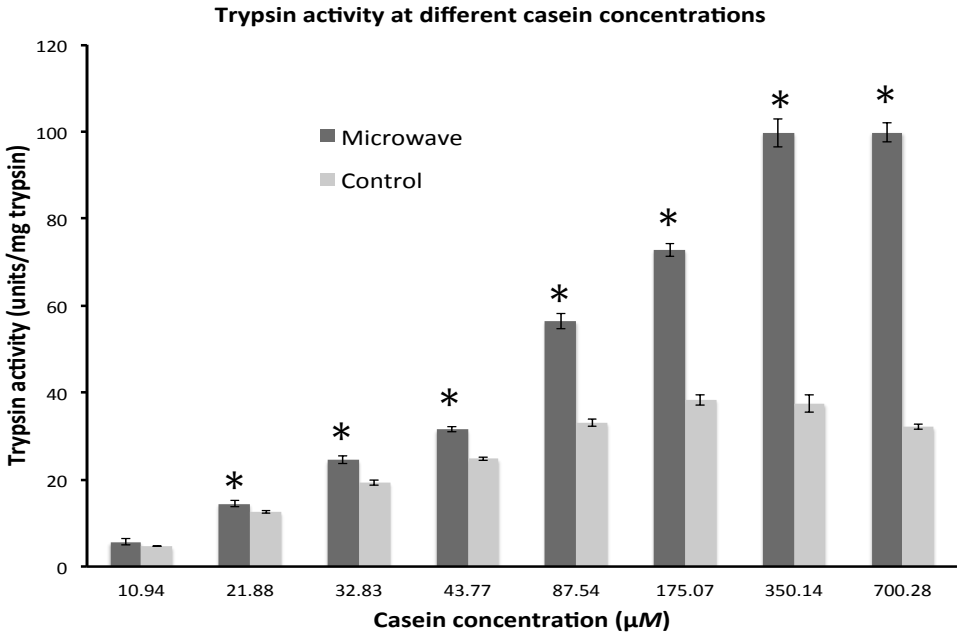

Figure 3.3. Trypsin activity determined at different casein concentrations. Data are triplicates for both control and microwave experiments. Microwave reactions were carried out with exposure to 9-10 W microwave while the temperature was maintained at 22°C. Definition of a unit: the amount in micromoles of L-tyrosine equivalents released from casein per minute. The flow rate of the CoolMate system in each experiment was manually adjusted to maintain constant bulk temperatures. Reformatted from the data reported previously,[17] with permission from Elsevier. Error bars are shown as standard deviation. Student's T-test, ∗ statistically significant difference, P-value < 0.01.

It should be emphasized that while performing the amylase and phosphatase experiments, attempts were made to confirm the trypsin results that were previously reported by us. Indeed, the increase of trypsin activity in the presence of 10 W microwave at constant bulk temperatures, *i.e.* 22°C, is reproducible. Considering the evidence presented in this work, it is quite clear that the impact of microwave on enzymatic activity, while reaction temperatures are maintained constant, is enzyme-dependent. While the

causes for these different behaviors is unknown, work in this lab is ongoing to further explore this phenomenon.

Acknowledgments

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(18) An assay described by Sigmaaldrich for the alkaline phosphatase from bovine intestinal mucosa is followed.

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Supplementary Material

Experimental procedures for this work can be found in the Supplementary Material.

Supplementary Material

Impact of microwave irradiation on enzymatic activity at constant bulk temperature is enzyme-dependent

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Note: All experiments were performed by Sina Atrin Mazinani.

Experimental

All chemicals and reagents were purchased from Sigmaaldrich without further purification. Milli-Q water was used for all experiments. A CEM Discovery Coolmate microwave system was operated at 2.45 GHz. Absorbance in the colorimetric assays was determined with a Biotek Powerwave xs Microtitre Plate Reader using a Gen5 software.

Comparison of temperatures measured by a fibre optic temperature probe and an alcohol-based glass thermometer

A CoolMate reaction vessel was charged with water (6.5 mL) and equipped with a fibre optic temperature probe and an alcohol thermometer, which were submerged in water in the reaction vessel to measure temperature simultaneously. The maximal microwave power was set at 10 W, while the target temperature of the reaction was set at 23°C, as reported by the fibre optic probe. While the reaction vessel was subjected to microwave irradiation, water in the vessel was cooled by circulating coolant (pre-cooled by liquid nitrogen) in the jacket in order to keep the temperature around 23°C. It was found that the temperatures reported by the fibre optic probe and thermometer were consistent over a period of one hour tested, within ±1 °C (Figure 3.1S).

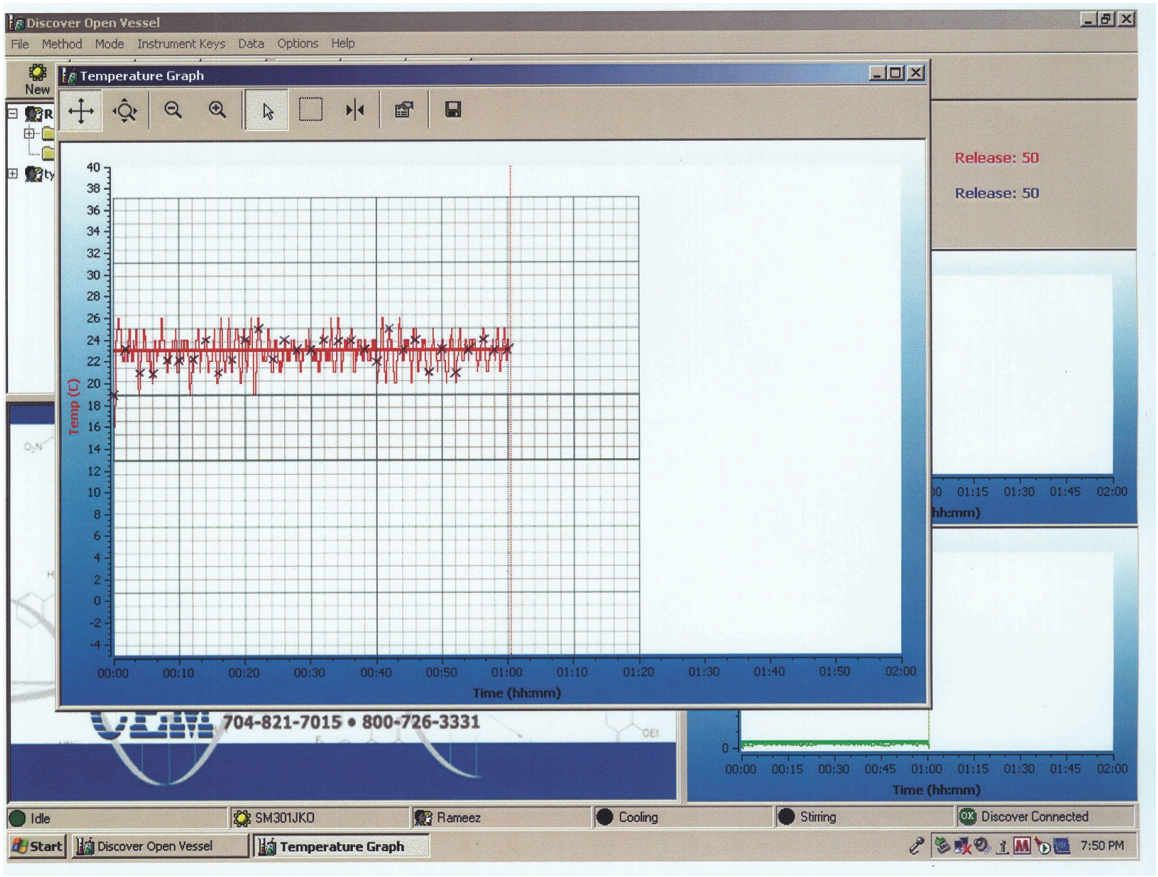

Figure 3.1S. Temperatures measured by a fibre optic temperature probe (Red line) and an alcohol-based glass thermometer (Black cross).

α-Amylase assay[1]

α-Amylase stock solution (25 µg/ml in water, α-amylase from *Bacillus licheniformis* lyophilized powder, Sigma A4551) was freshly prepared prior to each reaction. In each experiment 0.5 ml of *α*-amylase solution was added to 2.5 ml of starch stock solution (pH 6.9, starch from potato, Sigma S2004) of varying concentrations (Table 3.1S) containing sodium phosphate (20 m*M*) and sodium chloride (6.7 m*M*) in a microwave transparent (with negligible SAR) reaction vessel (10 mL Pressure Reaction Vial from CEM, Part Number 908035). After 5 min, 950 µl of the reaction mixture was withdrawn and mixed with 50 µl of NaOH (0.5 *M*) and 0.5 ml color reagent (prepared as described by Sigmaaldrich, http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-a-amylase.html, accessed on January 16, 2016) in an eppendorf tube. The eppendorf tube was incubated in a water bath at 90

°C for 15 min and was cooled down on ice for 3 min. Aliquot of the mixture (750 µl) was taken and diluted with water (2.25 ml) in a scintillation vial. Aliquots (270 µl) of the mixture were transferred to a 96-well quartz microtitre plate. Absorbance was read at 540 nm using a Biotek Powerwave xs plate reader.

Table 3.1S. Concentrations of starch solutions used in the experiments.

Starch concentration (%, or g/100 ml)	1.00	0.750	0.500	0.375	0.250	0.125

Control experiments were carried out in microwave reaction vessels (CEM, product number 908035) without microwave irradiation at 23 °C in a water bath on a hotplate stirrer. It is important to use the same reaction vessel as different reaction vessels/containers could lead to varying degrees of adsorption of enzyme on the surface.

Microwave experiments were carried out using a CEM Coolmate reactor. The temperature of the reaction mixture was kept constant at 23°C, as monitored by an alcohol glass thermometer that was inserted into the reaction mixture, by running coolant through the cooling jacket around the reaction vessel. In these experiments, microwave power was set and maintained at 10 W throughout the reaction. The flow of the coolant was adjusted manually such that the temperature of the reaction mixtures was maintained at 23°C.

Generation of maltose standard curve

Maltose stock solution (0.2%) was prepared. To various dilutions (Table 3.2S) of maltose (2.0 ml) were added color reagent (1.00 ml, prepared as described by Sigmaaldrich: http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-a-amylase.html, accessed on January 16, 2016) in eppendorf tubes. The eppendorf tubes were incubated in a water bath at 90 °C for 15 min and were cooled on icebox for 3 min. The eppendorf tube mixture (3.0 ml) was added to 9.0 ml water in a scintillation vial. Aliquots (270 µl) of the mixtures were added to a 96-well quartz microtitre plate. Absorbance was read at 540 nm using a Biotek Powerwave xs plate reader.

Table 3.2S. Concentrations of maltose dilutions.

Maltose concentration (%, or g per 100 ml)	0.2	0.1	0.08	0.06	0.04	0.02	0.005

Phosphatase Assay[2]

Phosphatase stock solution (0.1 μ*M*, alkaline phosphatase from porcine intestinal mucosa, Sigmaaldrich product number P7640) in glycine buffer (0.1 *M* glycine, 1 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$, pH 10.0) was freshly prepared prior to each reaction. For each experiment 50 μl of phosphatase solution was added to phosphatase substrate stock solutions (3.0 ml, 40 m*M* of 4-nitrophenyl phosphate disodium salt hexahydrate) of varying dilutions (Table 3.3S) in a microwave transparent reaction vessel. After 30 min aliquots (270 μl) of the mixture were withdrawn and added to a 96-well quartz microtitre plate. Absorbance was read at 405 nm using a Biotek Powerwave xs plate reader.

Table 3.3S. Concentration of phosphatase substrate dilutions used in experiments.

4-Nitrophenyl phosphate disodium salt hexahydrate concentration (m*M*)	40	26.67	20	10	5	2.5

Control experiments were carried out in a microwave transparent vessel (CEM, product number 908035) in water bath on a hotplate stirrer (without microwave irradiation) at 22°C.

Microwave experiments were carried out using a CEM Coolmate reactor. The temperature of the reaction mixture was kept constant at 22°C, as monitored by an alcohol thermometer that was inserted into the reaction mixture, by running coolant through the cooling jacket around the reaction vessel. In these experiments, microwave power was set and maintained at 10 W throughout the reaction. The flow of the coolant

was adjusted manually in such that the temperature of the reaction mixtures was maintained at 22°C.

Statistical analysis

Student's t-Test (Two-Tailed) was carried out in Excel. Experiments with p values not greater than 0.05 are considered as statistically significant. In each graph presented in the results, a significant difference is identified between samples.

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Chapter 4

Microwave irradiation of PC3 cells at constant culture temperature alters the incorporation of BODIPY into cells and reduction of MTT

Microwave, as a form of electromagnetic wave, has been widely used in heating and a range of applications such as in communications. While the literature in the past debated thermal and "non-thermal" aspects of microwave, recent literature focuses more on the "microwave-specific effects",[1-6] originating from the electromagnetic nature of microwave as a heating source that is fundamentally different from conventional heating through conduction.

We previously demonstrated effects of microwave irradiation under constant temperature conditions on certain protein activities.[7] The enzymatic activity of trypsin is significantly increased by exposure to 2.45 GHz microwave irradiation while maintaining temperature of the reaction mixture via simultaneous cooling. Interestingly though, the enzymatic activity of amylase and phosphatase are not affected by exposure to microwave under the same conditions,[8] indicating specificity of the effect.

While microwave is well known to harm cells when temperature increases are not prevented,[9-12] there is little information regarding potential effects of low power microwave, as might be encountered incidentally in some situations, on live cells under constant temperature conditions. Prorot and co-workers demonstrated that the membrane integrity of *Escherichia coli* was slightly altered in a power-dependent manner when the bacteria were subjected to pulses of microwave irradiation with powers up to 2000 W.[13] Ivanova and co-workers[14] exposed suspension cultures of bacteria, including Gram-positive cocci, such as *Planococcus maritimus*, *Staphylococcus aureus* and *S. epidermidis*, to 18 GHz microwave while temperature was allowed to fluctuate between 20 and 40ºC. These authors showed that bacterial cell membranes are permeabilized under these conditions.

In the present study we wished to follow up on these results by investigating the effects of low power (10 W) 2.45 GHz microwave irradiation on cell viability and cell membrane properties under conditions in which temperature is more strictly regulated at a biological optimum. We chose to focus on human cells since these are of particular interest from an environmental health perspective. We used PC3 human prostate cancer cells since they are tolerant of adherent growth (the initial propagation condition) and suspension growth (used during microwave exposure to accommodate instrument configuration). In order to maintain the culture temperature, the cultures were irradiated with microwave while the temperature, as measured in real-time by a fibre optic temperature probe (Figure 4.1), was maintained at 37±2ºC through cooling.

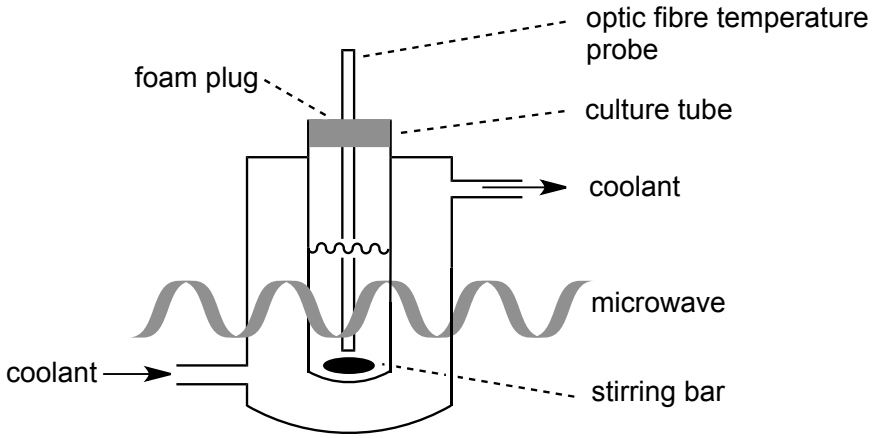


Figure 4.1. Schematic of the microwave irradiation setup.

Maintenance of temperature of PC3 cultures exposed to microwave: As demonstrated by us previously,[7,8] our system maintains temperature of the cell suspension in the CEM

microwave reactor within 1 or 2 degrees of the setpoint, here 37ºC. In the present study, temperatures of all the PC3 cell suspensions were continually monitored over the duration of the experiments. Sample output from the temperature monitor is shown in Figure 4.1Sa (Supporting Information), and microwave power output monitor in Figure 4.1Sb. Power output of 10 W was used, as this is at the lower end of the range of power that CEM Discovery microwave reactors generate.

Impact of microwave irradiation on PC3 cell viability: To determine whether 15 min of 10 W microwave irradiation at constant temperature was lethal to PC3 cells, we used two assays of cell death (phosphatidylserine, PS, externalization as a marker of apoptotic death, and propidium iodide, PI, staining as a marker of plasma membrane permeability associated with various forms of cell death) and one assay of cell viability (MTT assay measuring tetrazolium salt reduction by viable cells).

Plasma membranes maintain an asymmetry between bilayer leaflets, as most phospholipid biosynthesis takes place exclusively in the cytoplasm.[15] Spontaneous lipid flip-flop, or translocation, is extremely slow, with a half-life approximately several hours to days.[15-17] Translocation of PS to the outer membrane leaflet, usually enabled by flippases in an energy-dependent manner, is widely recognized as a signal in the early stage of apoptosis, as PS displayed on the outer membrane leaflet serves as a recognition marker for the cell to activate pro-inflammatory processes such as phagocytosis.[18-20] The Annexin V assay was used, since Annexin V irreversibly binds PS on the outer leaflet[21] and can be detected using flow cytometry. As shown in Figure 4.2a, the fluorescence intensities in both control and microwave-treated cultures are very low, and indeed only slightly higher than

autofluorescence (376 and 337 a.u. for control and microwave-treated cultures, respectively), indicating that microwave irradiation for 15 min under controlled temperature conditions had no effect on apparent PS externalization in PC3 cells.

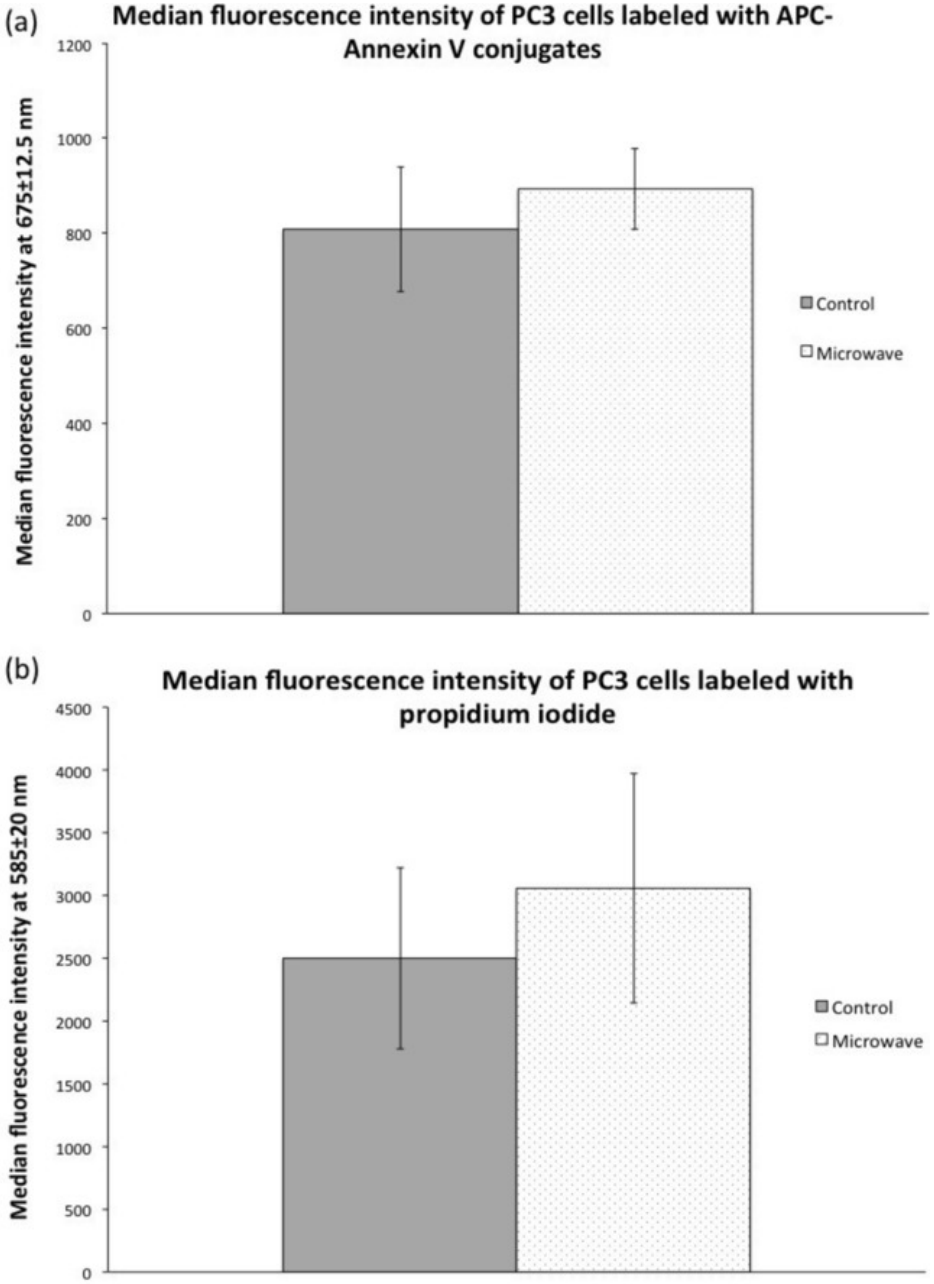


Figure 4.2. Comparison of PS flipping (a) and cell viability (b) between control and microwave-treated PC3 cells. (a). Median fluorescence intensity of APC at 675±12.5 nm; and (b). Median fluorescence intensity at 585±20 nm of PI. PC3 cells cultured in a water bath (control) at 37ºC or irradiated with 10 W (SAR: 700 mW/ml) microwave while temperature was maintained at 37ºC through simultaneous cooling. Replicates of three independent cultures. Error bars are shown as standard deviation.

We next assessed PI staining following the same microwave exposure protocol. PI is a cell impermeant fluorescent probe that is taken up in cells with damaged membranes and is thus routinely used to assay non-viable cells in which membrane integrity has been compromised.[22] We found no significant difference in PI staining between microwave irradiated and control cells (Figure 4.2b), and again the fluorescence intensities in both control and microwave-treated cultures are very low, and indeed only slightly higher than autofluorescence (975 and 688 a.u. for control and microwave-treated cultures, respectively), suggesting that microwave irradiation was not lethal to PC3 cells under the conditions used here.

The conclusion that microwave irradiation was not lethal to PC3 cells under the present experimental conditions was further supported by the MTT assays. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a cationic tetrazolium salt with hydrophobic moieties, is known to be membrane permeant, and is reduced to the corresponding (*E*, *Z*)-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan once inside the cell. The reduction of MTT is commonly used as a proxy of viable cell numbers.[23, 24]

MTT assay was carried out to compare viable cell counts in PC3 cells treated with microwave. As can be seen from Figure 4.3, formazan formation, as indicated by the absorbance normalized against cell density in the cells irradiated with microwave at 10 W while the temperature was maintained at 37ºC, is statistically significantly higher than that in the control experiments. As the absorbance is a measurement of the quantity of formazan present inside cells, which reflects the amount of MTT internalized by the cells, these results further confirmed that microwave treatment did not affect cell viability. As

suggested in the literature,[25] MTT is taken up by cells through endocytosis, in this respect, the increase in formazan in the cells treated with microwave could be a result of more efficient endocytosis facilitated by microwave irradiation. This hypothesis is currently being investigated.

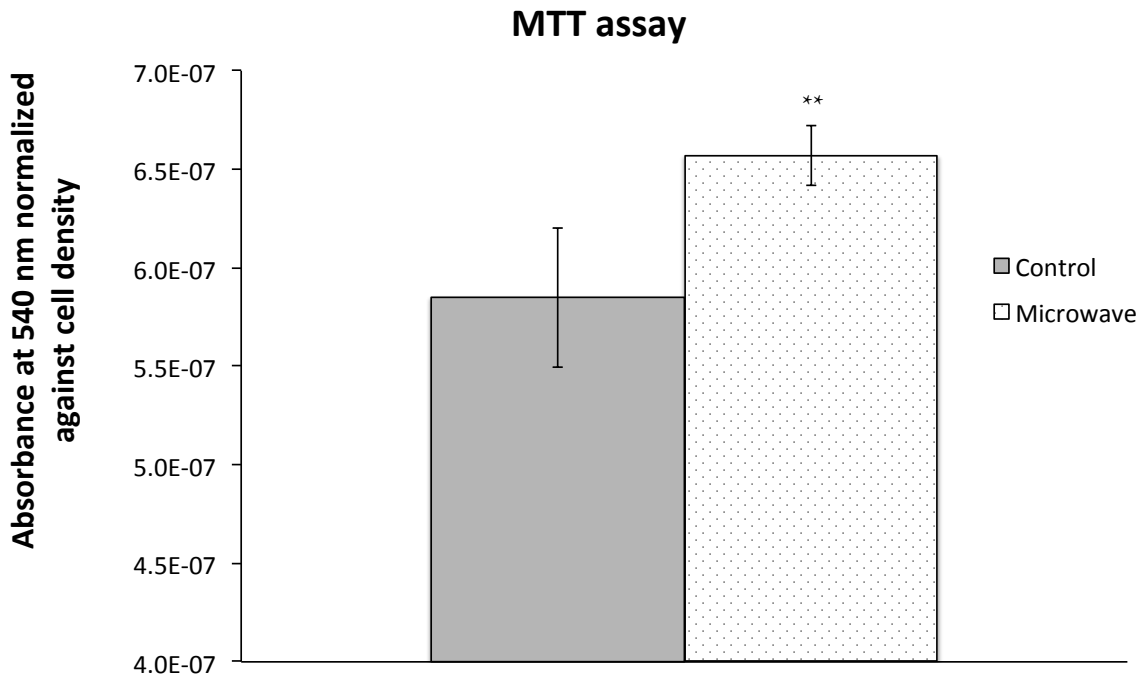
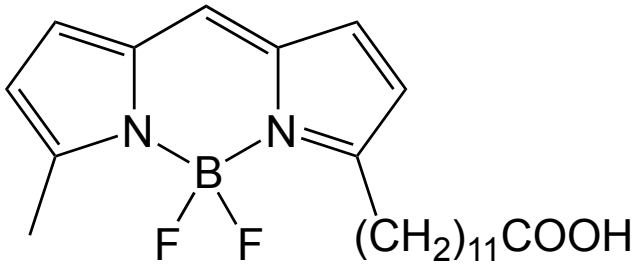


Figure 4.3. Absorbance at 540 nm in the MTT assay normalized against cell densities in PC3 cultures incubated at 37ºC in a water bath and irradiated at 10 W while the temperature was maintained at 37ºC through simultaneous cooling. Student's t-Test, ** $P < 0.02$. **.** Error bars are shown as standard deviation.

Incorporation of BODIPY into PC3 cells: BODIPY fatty acids have been demonstrated as useful membrane probes.[26,27] These fatty acids can be incorporated into cell membranes directly due to their amphiphilic nature, or undergo typical lipid transport and metabolism in cells.[28] In addition, BODIPY fluorophores are well known for their ability to form excimers, which causes a red shift in the fluorescence spectrum compared to the monomer form.[29-33] The BODIPY monomer and excimer fluorescence can therefore be used as a proxy of local BODIPY concentrations in cells.

In the present study, a BODIPY carboxylic acid (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid, BODIPY™ 500/510 dye, Life Technologies D3823) was added to PC3 cultures as an indicator for its incorporation into PC3 cells by quantifying fluorescence intensity of both monomers and excimers. Fluorescence of BODIPY™ 500/510 in PC3 cells was visualized by confocal fluorescence microscopy (Figure 4.4) and evaluated quantitatively using flow cytometry (Table 4.1).



BODIPY™ 500/510 dye

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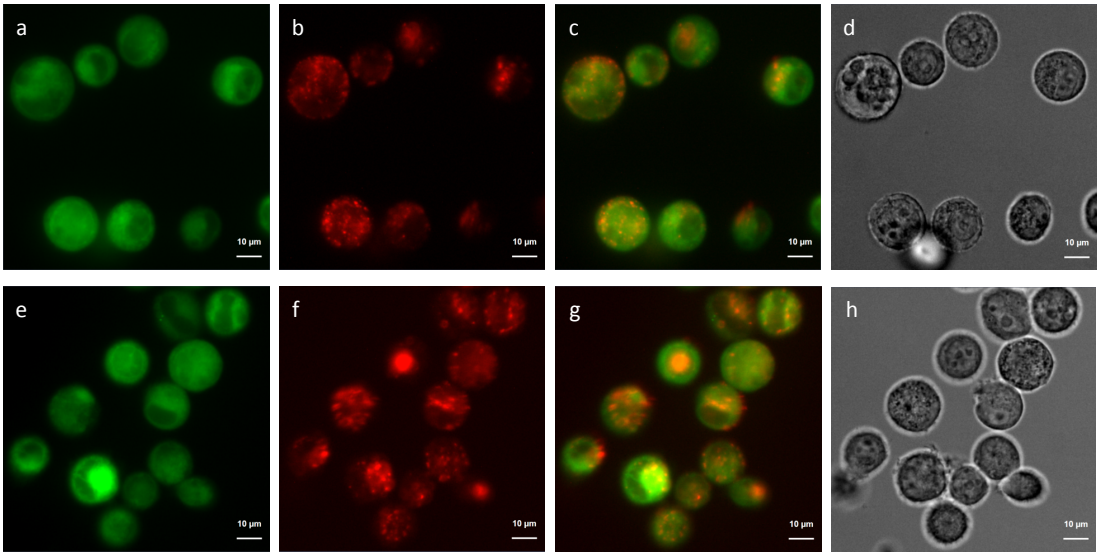

Figure 4.4. PC3 cells stained with BODIPY carboxylic acid. (a and e). Fluorescence images of PC3 cells in the green channel (Ex. 470±20, Em. 525±25); (b and f). Fluorescence images of PC3 cells in the red channel (Ex. 560±20, Em. 630±37.5); (c and g). Overlay of green and red channel; (d and h). Differential interference contrast (DIC) images of PC3 cells. Images a–d: control cells incubated at 37ºC. Images e–h: cells irradiated with 10 W microwave while maintaining temperature at 37ºC.

Confocal microscopic images shown in Figure 4.4 confirmed that the BODIPY carboxylic acid does indeed fluoresce both green and red in the PC3 cells, representing emission from BODIPY monomers and excimers, respectively. Flow cytometry revealed that green and red fluorescence intensities are statistically higher in the cells treated with microwave (Table 4.1). While the ratios of emission intensities in the green and red channel are virtually the same for the control and microwave-treated PC3 cells, a significant and consistent increase in fluorescence intensities in the cells exposed to microwave irradiation was observed, suggesting that incorporation of BODIPY carboxylic acid into the cells exposed to microwave irradiation was more efficient, presumably due to the perturbation of cell membranes by microwave irradiation.

Table 4.1. Fluorescence intensity counts of PC3 cells labelled with BODIPY carboxylic acid. [a]P values were calculated for median fluorescence intensity 1 and 2 for FL1 and FL3 channels, respectively (Student's t-Test).

	FL1 533±15 nm	FL3 610±10 nm	Ratio of intensity of monomer/excimer emission
Median fluorescence intensity 1 (PC3 cells irradiated with microwave while temperature kept at 37ºC)	2,153,921	14,130	152
Median fluorescence intensity 2 (PC3 cells incubated at 37ºC in a water bath)	1,392,106	9,753	143
Percentage difference in median fluorescence intensity (Fluorescence intensity 1–Fluorescence intensity 2)/ Fluorescence intensity 2	55%	45%	-
P-value[a]	0.02	0.002	-

Conclusions

While the current literature is divided in the existence of potential "microwave-specific effects" where the unique electromagnetic nature of microwave has been under debate for its influence on biological systems, the present study investigated the potential impact of microwave irradiation on cell viability and membrane properties in a PC3 cell line model. This study represents an investigation where the temperature of the cell cultures is maintained virtually constant throughout the experiment. This criterion is important, as results would have been meritless if the culture temperature was allowed to rise as a result of microwave heating. Results from this study indicated no effect of constant temperature microwave on cell viability, but suggest potential perturbation of PC3 cell membrane when exposed to microwave irradiation at constant temperature. Considering the electromagnetic nature of microwave, and the fact that the culture temperatures are well controlled in the present investigation, these observations suggest that perturbation of membrane properties in the presence of microwave irradiation reflects effects not caused by conventional heating through conduction. While it is tempting to compare membrane perturbation by microwave irradiation to electroporation by electric pulses,[34, 35] which is a well-established phenomenon and technique, detailed studies on the influence of microwave irradiation on cell membrane potentials will be necessary in order to establish the theoretic basis for this comparison. Furthermore, future work will investigate possible consequences of alteration of cell membrane properties, such as cell morphology, and differential gene expressions upon exposure to non-lethal microwave irradiation.

Experimental Section

Note: All experiments were performed by Sina Atrin Mazinani.

Buffers: Modified DMEM: Dulbecco's Modified Eagle Medium containing 10% Fetal bovine serum, 2% Non-essential amino acids, 1% penicillin/streptomycin. Perfusion buffer: NaCl 101 mM, KCl 5 mM, MgCl2 2 mM, CaCl2 2 mM, D-glucose 5 mM, D-mannitol 50 mM, Hepes-Tris 5 mM, pH 7.4. The buffer was autoclaved at 121 °C for 30 min.

PC3 cell cultures: PC3 human prostate cancer cells were cultured in modified DMEM in 10 cm cell culture dishes (Sarstedt) until a confluent monolayer of adherent cells was obtained. The cells were washed twice with 1×PBS (4.0 ml, pH 7.4). Trypsin-EDTA solution (3 ml, 0.25%, Sigma, T4049) was then added and the plate was incubated at 37 °C for 3 min in a culture incubator (20% O2, 5% CO2, 37°C). DMEM (4.0 ml) was then added to the plate and the cell suspension was transferred to a 15 ml Falcon tube and centrifuged at 970 g for 3 min to obtain a cell pellet, which was used for subsequent experiments.

Microwave irradiation set up: A CEM Discovery Coolmate microwave system running at 2.45 GHz was used for the irradiation of PC3 cell cultures. The microwave reactor was controlled with Synergy software (CEM). PC3 cultures were incubated in CEM standard reaction vessels (catalogue number 168302), together with the CEM attenuator assembly (catalogue number 542476) that allows for the circulation of coolant around the culture tube to maintain the temperature of cultures. Solvay Solexis H Galden Zt 130 coolant was pre-cooled in the Coolmate coolant reservoir by liquid nitrogen. During experiments, the culture temperature was maintained at 37±2ºC by adjusting the temperature of coolant and

flow rates, and monitored with a fibre optic temperature probe in real time. Cultures were mixed by stirring with a stirring bar (1×8 mm), with the stirring setting at low (540 revolution per minute as determined by an Omega HHT13 Tachometer). The experimental setup is illustrated in Figure 4.1.

Confocal microscopy: Fluorescent images of PC-3 cells were obtained using a Carl Zeiss Axio Observer. The microscope stage temperature was kept at 37 °C under 5% CO2. Both BODIPY and Annexin V-APC probes were included in the cell suspension throughout the microwave treatment and control experiments. For BODIPY assay, green fluorescence of BODIPY was detected using a 470±20 nm excitation and 525±25 nm emission filter set. Red fluorescence of BODIPY (excimer) was detected using a 560±20 nm excitation and 630±37.5 nm emission filter set. For Annexin V-APC assay, red fluorescence of Annexin V-APC was detected using a 620±30 nm excitation and 700±37.5 nm emission filter set. Red fluorescence of PI was detected using a 545±12.5 nm excitation and 605±35 nm emission filter set. The intensity of fluorescence excitation by an X-Cite 120LED light source and camera exposure times were held constant across experiments.

Flow cytometry: Median (and mean) fluorescence intensity of stained PC3 cells were obtained using a BD ACCURI C6 flow cytometer. For BODIPY assay, green fluorescence of BODIPY was measured in Fl-1 channel (Em 533±15 nm). Red fluorescence of BODIPY (excimer) was measured in Fl-3 (Em 610±10 nm). For Annexin V-APC assay, red fluorescence of Annexin V-APC was measured in Fl-4 channel (Em 675±12.5 nm) and red fluorescence of propidium iodide was detected in Fl-2 channel (Em 585±20 nm).

Annexin V-APC assay to compare the flipping of phosphatidylserine: The PC3 cell pellet obtained from the culture was resuspended in DMEM and cell density was

determined using a hemocytometer. Annexin V-APC (10 µl, eBioscience, 88-8007-72) was added to the cell suspension and gently swirled for 15 seconds. The cell suspension was then divided into two 1.8 ml portions, each of which was transferred to a 9 ml microwave reaction vessel (CEM, catalogue number 168302), one for microwave treatment and another control. One tube of control cells was incubated at 37 °C in a water-bath on a hotplate and mixed with a stirring bar (1×8 mm) at 540 rpm. The other tube containing PC3 cells was placed in a CEM microwave reactor and exposed to microwave irradiation at 10 W and 37°C, while being stirred at the low speed setting (540 rpm), and temperature of the cell suspension was maintained at 37 °C through simultaneous cooling. After 15 min, 990 µl of each sample (microwave and control) was transferred to an eppendorf tube, followed by the addition of propidium iodide (PI, 10 µl, 1 mg/ml) to each eppendorf tube. The tubes were incubated at 25 °C for 10 min. The tubes were centrifuged at 5000 g for 3 min and the cell pellet was resuspended in 1.0 ml 1×PBS prior to flow cytometry. The median average fluorescence intensity of each sample, as well as autofluorescence, was determined in the Fl-4 (Em 675±12.5 nm) and Fl-2 (585± 20 nm) channels for Annexin-V and PI, respectively.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay: PC3 cell pellets were resuspended in 6.0 ml 1×PBS and the cell density was determined with a hemocytometer. A solution of MTT (0.6 ml, 5 mg/ml) was added to the cell suspension. The cell suspension was divided into two equal 3.0 ml volumes and transferred into two 9 ml microwave reaction vessels (CEM, 168302), one for microwave treatment and another control as described above. After 20 min, 2.0 ml of each sample was transferred to a 15 ml falcon tube and centrifuged at 970 g for 5 min. After decanting the supernatant, the cell

pellet was dissolved in DMSO (0.6 ml) and incubated at 37 °C in a static incubator for 10 min. Aliquots (100 µl) from the microwave-treated and control samples were transferred to a 96-well plate and the absorbance was read at 540 nm.

BODIPY assay: PC3 cell pellet was resuspended in 6.0 ml labeling solution (4.0 ml DMEM, 1.466 ml perfusion buffer, 0.534 ml 1% pluronic buffer) and cell density was determined with a hemocytometer. A solution of 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY™ 500/510 dye, Life Technologies D3823) in DMSO (4 µl, 1 mg/ml) was added to the cell suspension. Two 2.9 ml portions of the cell suspension were transferred to two 9-ml microwave reaction vessels (CEM, 168302) and treated as described above. After 20 min, an aliquot (1.0 ml) of each sample was transferred to an eppendorf tube and centrifuged at 5000 g for 3 min. After decanting the supernatant, the cell pellet was resuspended in 1.0 ml 1×PBS and analyzed by flow cytometry. The median fluorescence intensity of each sample was obtained in two fluorescent channels, Fl-1 (Em 533±15 nm) and Fl-3 (Em 610±10 nm), for the fluorescent emission of BODIPY monomer and excimer, respectively. Another aliquot (200 µl) of the cell suspension was taken for imaging by confocal microscopy at EGFP (green), mcherry (red) and DIC channels.

Statistical analysis

Two-Tailed t-Test was carried out in Excel. Experiments with p values not greater than 0.05 are considered as statistically significant. In each graph presented in the results, a significant difference is identified between samples.

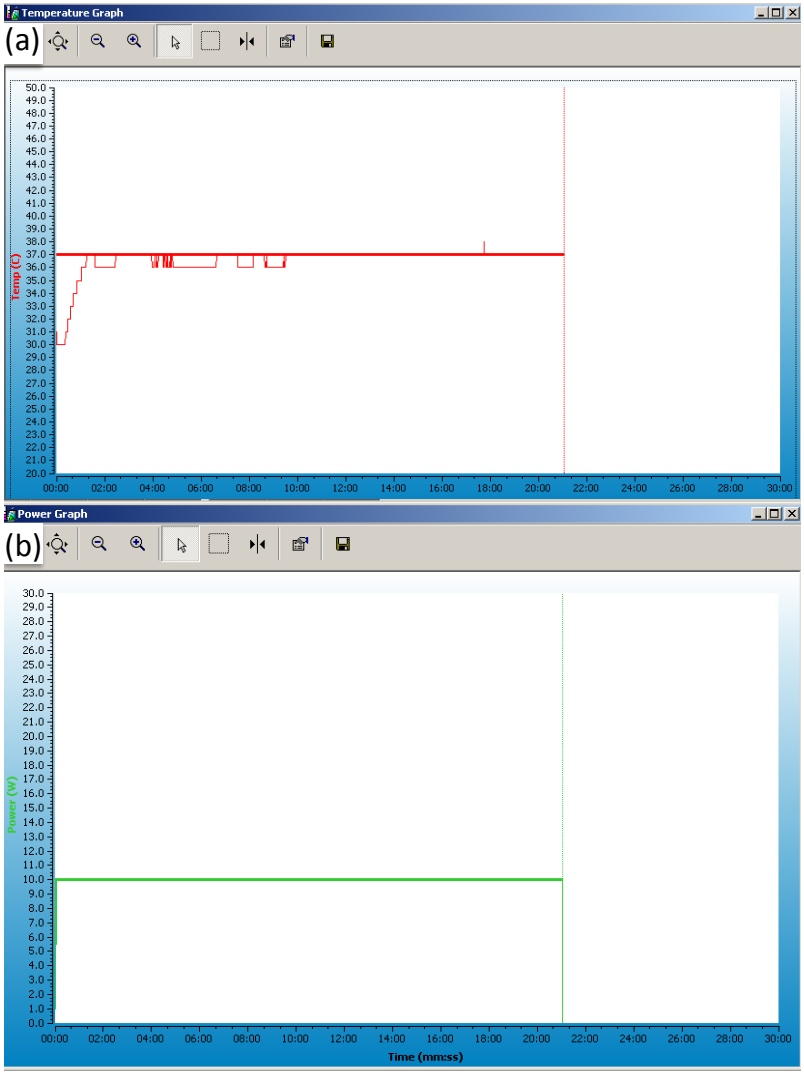

Figure 4.1S. Screenshots of (a) culture temperature and (b) microwave power of PC3 cultures exposed to microwave irradiation.

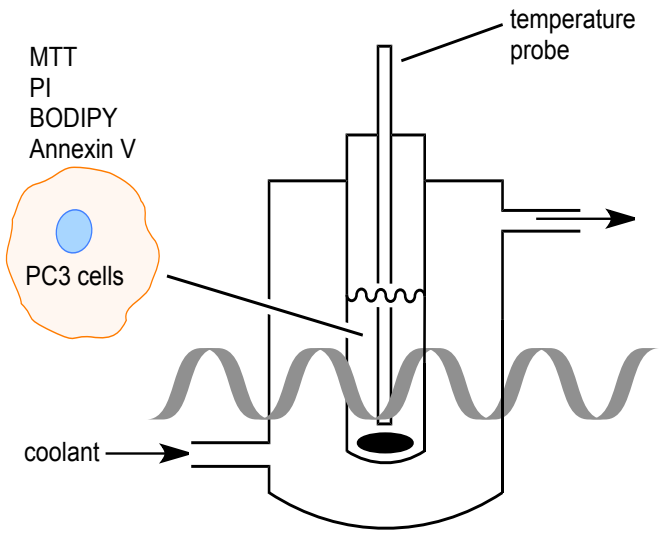

Figure 4.2S. Schematic of the microwave irradiation setup.

Acknowledgements

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Keywords: Apoptosis • BODIPY membrane probe • cell viability • microwave irradiation • MTT assay • PC3 human prostate cancer cells

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Chapter 5

Microwave-assisted delivery of an anticancer drug to cancer cells

In the last few decades, microwave has been recognized for its unique properties in transmitting energy as a heating source and passage of signals, however, there has been some controversy surrounding the safety of microwave exposure. Much of the recent investigation on microwave as a heating source focuses on the existence of "microwave-specific effects",[1,2] that is, possible aspects of microwave that are not associated with heating. We have explored several of these microwave-specific effects by studying the behaviours of biological systems exposed to microwave while temperature was carefully controlled through simultaneous cooling. Using this approach, we demonstrated that low power microwave can affect enzyme activities. Trypsin, but not α-amylase or phosphatase, activity was significantly increased by exposure to 10 W microwave at relatively constant temperature.[3,4] Low power microwave also appears to slightly perturb membrane properties in a PC-3 prostate cancer cells, without causing apoptosis or necrosis.[5] *Escherichia coli* growth is also affected by exposure to non-lethal microwave, though the effect is transient (unpublished results). Taken together, our findings suggest that exposure of cells to low power microwave at constant temperatures causes subtle perturbations to cells without inducing apoptosis.

Based on our work with PC-3 human prostate cancer cell we hypothesize that exposure of cells to non-lethal microwave irradiation at constant temperature transiently disrupts cell membrane permeability and/or stimulates endocytosis, promoting uptake of extracellular molecules. This scenario resembles electroporation, but with tissue penetration depth (2–4 cm)[6] that is quite relevant with many tumors. It is therefore possible to use microwave (or radiofrequency) to promote internalization of therapeutic

agents into cells. In the present communication, we report significantly increased cellular uptakes of the anticancer drug doxorubicin by MCF-7 human breast and PC-3 human prostate cancer cells, associated with reductions in cancer cell viability.

MCF-7 breast cancer cells were cultured in a CEM Coolmate microwave reactor at 37°C with 26 μM doxorubicin. Temperature was monitored with a fibre optic temperature probe and constant temperature was maintained through simultaneous cooling (see Figure 4.1S for representative temperature and microwave power profiles for cultures in the microwave reactor). As demonstrated previously,[3,4] we were able to maintain the temperature of the culture within ±1-2 °C of setpoint values.

Two experiments were carried out at 37°C in the presence of doxorubicin, one where MCF-7 cells were grown without microwave, while in the other MCF-7 cells were exposed to 10 W microwave. After 5, 10 and 20 minutes, the cultures were pelleted by centrifugation, washed and subsequently analyzed by flow cytometry to compare the level of doxorubicin internalization based on its inherent fluorescent property. As Figure 5.1a shows, the uptake of doxorubicin was significantly increased by exposure to microwave in a time-dependent manner. The uptake of doxorubicin by MCF-7 cells treated with microwave for 20 minutes was 108% higher than that of the control culture. Figures 5.1c and 5.1d show the fluorescent confocal microscopic images of MCF-7 cells treated with doxorubicin alone and doxorubicin in the presence of microwave irradiation, respectively. Doxorubicin was clearly localized in the nucleus, consistent with literature observations.[7]

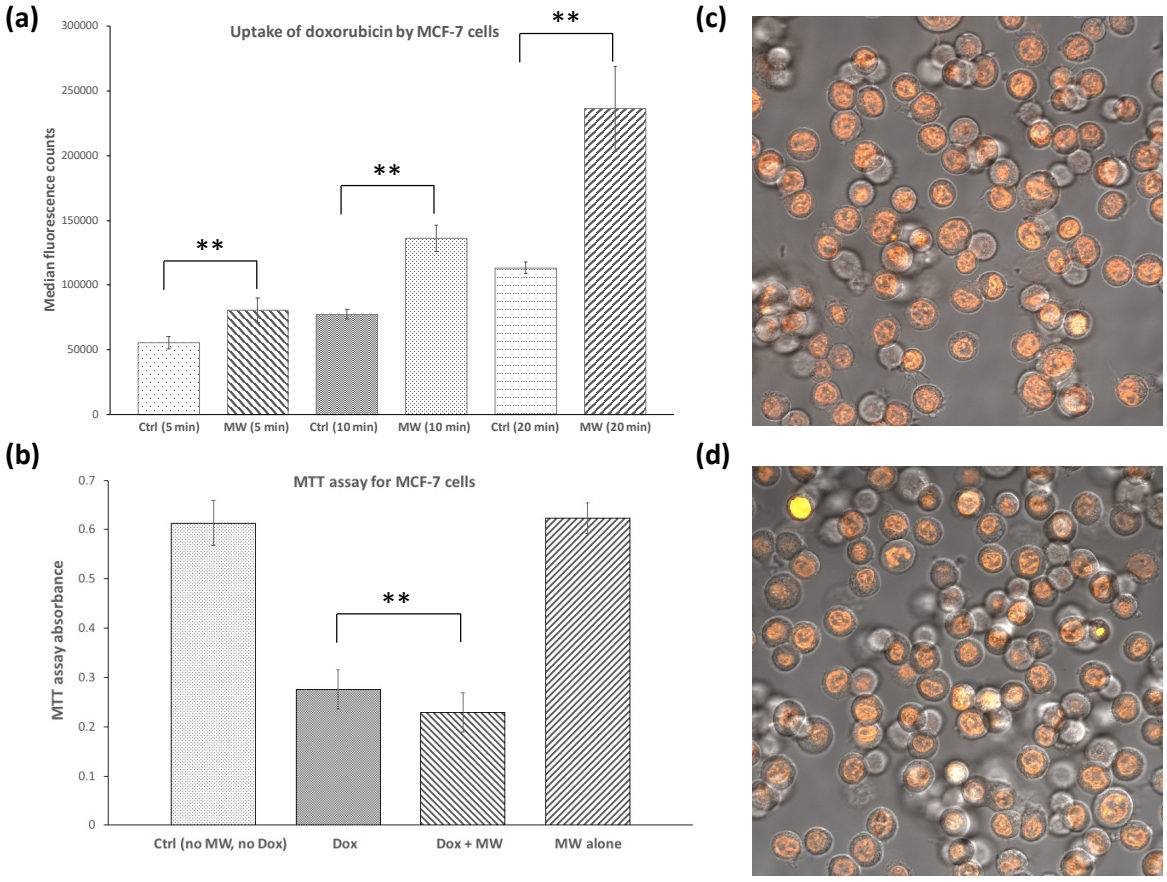

Figure 5.1. MCF-7 cells cultured under different conditions. (a). Microwave-assisted internalization of doxorubicin (Median fluorescence at 610±10 nm). Experiments were carried out at 5, 10 and 20 minutes. (b). MTT viability assays of MCF-7 cells treated with doxorubicin and microwave for 20 minutes and then cultured for additional 24 hours before MTT assays were conducted (absorbance at 540 nm). (c). Fluorescent confocal image of MCF-7 cells after incubation with doxorubicin for 20 minutes. (d). Fluorescent confocal image of MCF-7 cells after incubation with doxorubicin for 20 minutes in the presence of 10 W microwave. **P<0.005 for biological triplicate experiments. Error bars are shown as standard deviation. Student's t-Test (two-tailed) was performed for statistical analysis.

In order to demonstrate the anticancer activity of internalized doxorubicin, MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assays were conducted.[8] Thus, MCF-7 cell cultures were incubated under four conditions, (1) in the absence of doxorubicin, (2) in the presence of 26 μM doxorubicin, and (3) in the presence of 26 μM doxorubicin and exposed to 10 W microwave, and (4) exposed to 10 W microwave only, all at 37°C. After 20 minutes, microwave exposure was discontinued, and all four cultures were incubated for 24 h, followed by standard MTT assay quantification. Figure 5.1b showed that cell viability was not affected by exposure to microwave for 20 minutes alone, while reduced by 55 and 63% in cultures (2) and (3), respectively. This set of experiments clearly demonstrated the increased anticancer activity when microwave is administered together with doxorubicin.

Similar results were obtained when PC-3 human prostate cancer cells were treated with doxorubicin while exposed to microwave irradiation (Figure 5.2Sa in the supplementary material). Under the same conditions as described above for MCF-7 cells, uptake of doxorubicin by PC-3 cells was increased by 73% in the presence of microwave for 20 minutes. Cell viability as indicated by MTT assays was reduced by 25 and 40% for PC-3 cells treated with doxorubicin alone and doxorubicin in the presence of microwave, respectively (Figure 5.2Sb in the supplementary material). Again, treatment of PC-3 cells with microwave alone did not lead to significant reduction in cell viability as indicated by the MTT assay.

Doxorubicin is believed to enter cells by passive diffusion,[9,10] however, internalization of doxorubicin through endocytosis has also been suggested, especially when it forms a complex or conjugate with another substance.[11,12,13] To determine whether endocytosis is involved in the microwave-promoted uptake, inhibitors of this pathway were included during experiments. Thus, Pitstop2[14] and Dynasore[15] that are known inhibitors for clathrin-dependent and dynamin-dependent endocytosis, respectively, were added to cell cultures 30 minutes prior to treatment with doxorubicin. Doxorubicin uptake was subsequently measured by flow cytometry. As can be seen in Figure 5.2, no effect of Pitstop2 or Dynasore on the uptake of doxorubicin in the absence of microwave was observed, indicating that endocytosis does not contribute to doxorubicin uptake under our cell culture conditions. Similarly, when MCF-7 cells were pre-treated with Pitstop2 and Dynasore for 30 minutes, followed by doxorubicin in the presence of microwave there was no decrease in doxorubicin uptake (Figure 5.2). These results suggest that the enhanced uptake of doxorubicin facilitated by microwave is not via endocytosis.

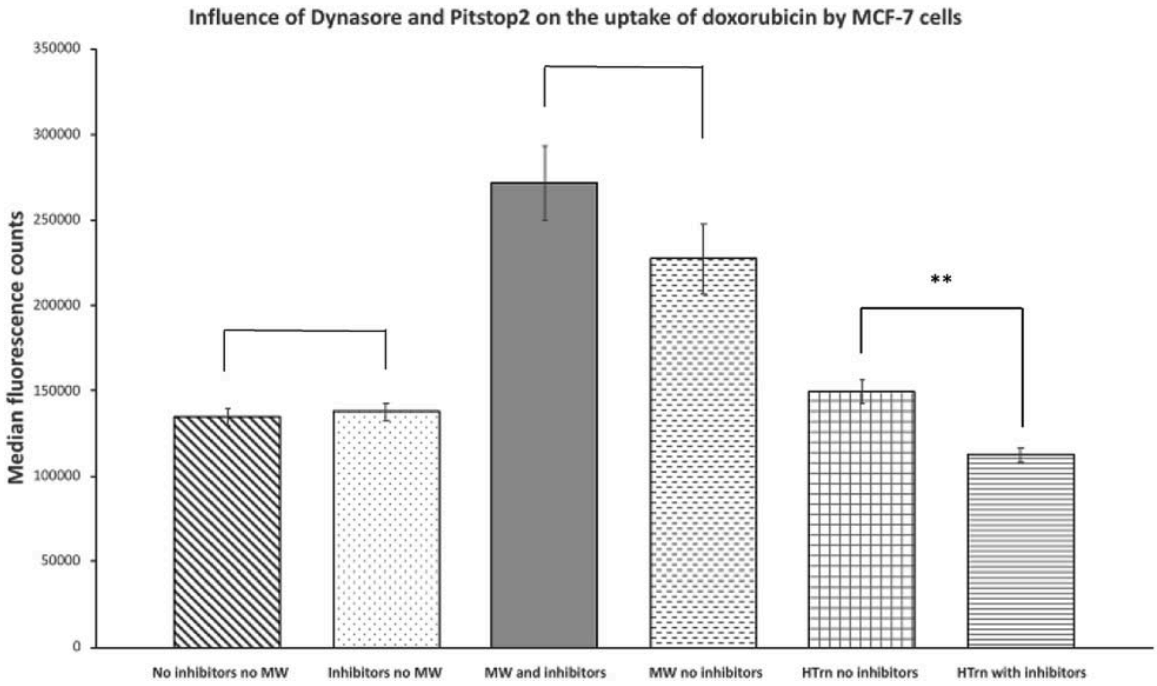

Figure 5.2. Influence of endocytosis inhibitors Dynasore and Pitstop2 on the uptake of doxorubicin by MCF-7 cells. Inhibitory effects of Dynasore and Pitstop2 on the uptake of Human transferrin 488A by MCF-7 cells which occurs through endocytosis (positive control). Experiments were carried out in pairs as indicated by the bars as biological triplicates. **$P < 0.05$. Error bars are shown as SEMs calculated from 3 to 12 separate measurements ($n = 3–12$).

To further rule out the involvement of endocytosis in the microwave-assisted internalization of doxorubicin, we measured uptake of a fluorescently labeled oligodeoxyribonucleotide (FAM-5'-TTG GTG GAT AGT TAT TAG-3', FAM: fluorescein) by MCF-7 cells in the presence and absence of microwave exposure. While the uptake of oligonucleotides by cells is complex, it is believed to involve endocytosis.[16] Results indicate (Figure 5.3S in the supporting material) that microwave did not increase uptake of the oligonucleotide by MCF-7 cells. While previous work[17] reported that

treatment of cultured myoblasts with rather high power microwave (420 W in pulses, as opposed to 10 W used in the present study) led to enhanced delivery of 2'-*O*-methylphosphorothioate antisense oligonucleotides, cells exposed to such a significant microwave power are likely to experience notable alteration in cellular structures and functions; as such, no comparison can be drawn between the present work and the literature precedence.

Taken together, results from both MCF-7 and PC-3 cancer cell culture experiments support the notion that uptake of anticancer drugs such as doxorubicin by cells is promoted by exposure to non-lethal microwave. It is of interest to note that the promotion of doxorubicin internalization by microwave is dependent on the duration of exposure, while exposure to microwave alone does not lead to changes in cell viability as evidenced by the MTT assay results. These observations are in agreement with our previous finding that suggested disturbance of cell membranes by non-lethal microwave. In this respect, it is likely that exposure of cells to non-lethal microwave irradiation perturbs cell membranes, allowing for more efficient internalization of doxorubicin, much in the same manner as electroporation. This work strongly supports a novel microwave (radiofrequency)-assisted drug delivery platform to improve the efficacy of chemotherapy, with potential to reduce side effects. As side effects and efficacy are the major challenges in cancer chemotherapy, strategies that can address both are valuable. By targeting tumors directly with microwave radiation to stimulate drug uptake it may be possible to lower the drug dose, thus limiting side effects. No adverse effects associated with low power microwave have ever been identified, and higher power microwave is

already used clinically for tissue ablation.[18] Therefore, there is an opportunity for rapid uptake of this technique into the clinic. Future work will examine the mechanistic aspect of the microwave-promoted uptake. The optimal frequency, power and duration of microwave will also be profiled.

Acknowledgements

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Author Contributions

S.A.M. contributed to experimental design and performed all the experiments. J.A.S. advised on cell culture experiments. H.Y. contributed to the conceptualization of the project, experimental design, and wrote the manuscript. All authors discussed the results and contributed to the writing and revision of the manuscript.

Competing financial statement

The authors declare no competing financial interests.

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Supplementary material

Microwave-assisted delivery of an anticancer drug to cancer cells

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Note: All experiments were performed by Sina Atrin Mazinani.

ONLINE METHODS

Media: Modified DMEM (Dulbecco's Modified Eagle Medium) contains 10% Fetal bovine serum, 2% non-essential amino acids, 1% penicillin/streptomycin.

MCF-7 and PC-3 cell cultures: MCF-7 human breast cancer cells and PC-3 human prostate cancer cells were cultured in modified DMEM in 10 cm cell culture dishes (Sarstedt) until a confluent monolayer of adherent cells was obtained. The cells were washed twice with 1×PBS (4.0 ml, pH 7.4). Trypsin–EDTA solution (3 ml, 0.25%, Sigma, T4049) was then added and the plate was incubated at 37 °C for 3 min in a culture incubator (20% O_2, 5% CO_2, 37°C). DMEM (4.0 ml) was then added to the plate and the

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cell suspension was transferred to a 15 ml Falcon tube and centrifuged at 970 g for 3 min to obtain a cell pellet, which was used for subsequent experiments.

Microwave irradiation set up: A CEM Discovery Coolmate microwave system running at 2.45 GHz was used for the irradiation of MCF-7 and PC-3 cell cultures. The microwave reactor was controlled with Synergy software (CEM). MCF-7 and PC-3 cultures were incubated in CEM standard reaction vessels (catalogue number 168302), together with the CEM attenuator assembly (catalogue number 542476) that allows for the circulation of coolant around the culture tube to maintain the temperature of cultures. Solvay Solexis H Galden Zt 130 coolant was pre-cooled in the Coolmate coolant reservoir by liquid nitrogen. During experiments, the culture temperature was maintained at 37±2°C by adjusting the temperature of coolant and flow rates and monitored with a fibre optic temperature probe in real time. Cultures were mixed by stirring with a stirring bar (1×8 mm), with the stirring setting at low (540 revolution per minute as determined by an Omega HHT13 Tachometer).

Confocal microscopy: Fluorescence images of MCF-7 and PC-3 cells were obtained using a Carl Zeiss Axio Observer microscope. The microscope stage temperature was kept at 37 °C under 5% CO_2. For doxorubicin assay, red fluorescence of doxorubicin was detected using a 545±12.5 nm excitation and 605±35 nm emission filter set. The intensity of fluorescence excitation by an X-Cite 120LED light source and camera exposure times were held constant across experiments.

Flow cytometry: Median fluorescence intensity of stained MCF-7 and PC-3 cells were obtained using a BD ACCURI C6 flow cytometer. In doxorubicin assay, red fluorescence

of doxorubicin was measured in Fl-3 channel (Em 610±10 nm). Number of events recorded was 100,000 for all experiments and FSC-H threshold was set to 80,000.

Doxorubicin assay (MCF-7 and PC-3 cells)

Cell density was measured by a hemocytometer. By dilution, cell density was adjusted to ~500,000 per ml prior to each experiment. To a 6 ml cell suspension in modified DMEM was added 10 µl of doxorubicin (1 mg/ml) to give a final concentration of 26 µM. Two aliquots of 3 ml cell suspension were transferred to the reaction vessel in CEM (for microwave treatment) and a reaction vessel in a water bath on hotplate (control treatment).

CEM reaction vessel parameters were adjusted to 37°C, maximum power 10 W, and slow stirring (540 rpm). For control, the temperature of the water bath was pre-set at 37°C and the stirring speed of hotplate was set to 540 rpm. Samples were treated for 20 minutes under each condition. At the end of each treatment, 4 samples of 0.75 ml were taken from each reaction vessel (MW and control) and were transferred to microcentrifuge tubes. The microcentrifuge tubes were spun at 2300 rcf for 3 min to obtain cell pellets. After aspirating the supernatant, the cell pellets were re-suspended in 1×PBS (1 ml) and were centrifuged for 3 min at 2300 rcf. The supernatant was discarded, and the cell pellet was re-suspended in 0.75 ml 1×PBS. The samples were analyzed by a flow cytometer (BD Accuri) and the median fluorescence of each sample at FL-3 channel was recorded.

Fluorescent images were obtained in channels mcherry (Ex: 560±20 nm, Em: 630±37.5 nm) and cy3 (Ex: 545±12.5 nm, Em: 605±35 nm).

MTT assay

Cell density was measured by a hemocytometer. By dilution, cell density was adjusted to ~500,000 per ml prior to each experiment. To a 6 ml cell suspension in modified DMEM was added 10 µl of doxorubicin at 1 mg/ml to reach a final concentration of 26 µM. Cell suspensions (3 ml) were transferred to the reaction vessel in CEM (for microwave treatment) and a reaction vessel in a water bath on hotplate (control treatment). As a negative control, to a 3 ml cell suspension at similar cell density (500,000 per ml) was added 5 µl DMSO which was transferred to a reaction vessel in water bath.

CEM reaction vessel parameters were adjusted to 37°C, maximum power 10 W, and slow stirring (540 rpm). For control, the temperature of the water bath was previously set to 37°C and the stirring speed of hotplate was set to 540 rpm. Samples were treated for 20 minutes under each condition. At the end of each treatment, samples of 1 ml from each reaction vessel (MW and control) were transferred to microcentrifuge tubes. The microcentrifuge tubes were spun at 2300 rcf for 3 min to obtain cell pellets. After aspirating the supernatant, the cell pellets were re-suspended in modified DMEM (1 ml) and were centrifuged for 3 min at 2300 rcf. The supernatant was discarded, and the cell pellet was re-suspended in modified DMEM (1 ml). The cell suspension was then transferred to counting plates (SARSTEDT, TC Dish, REF 83.3901.002) containing 3 ml modified DMEM.

The plates were incubated for 24 hours in an incubator (5% CO_2, 20% O_2 and 37°C) After the 24-hour incubation, cells were trypsinized and then re-suspended in 1 ml DMEM buffer. To the cell suspension was added 100 µl of MTT (5 mg/ml) in a 15 ml falcon tube. After incubation at 37°C for 30 minutes, the cell suspension was centrifuged at 4255 rcf for 3 min. The supernatant was discarded, and the cell pellet was dissolved in 0.5 ml DMSO, followed by incubation at 37°C for 10 minutes. Aliquots of 100 µl of the solution were transferred to a 96-well microtiter plate and the absorbance was recorded at 540 nm by a plate reader (Bio-Tek PowerWave Microplate Spectrophotometer).

MTT assay for control to investigate the effect of microwave treatment on cells in the absence of doxorubicin

Same MTT assay protocol as described above was followed, except that instead of doxorubicin, 10 µl of filter-sterilized DMSO was used.

Pitstop 2 and Dynasore assay

To a 6 ml cell suspension of MCF-7 cells at a density of 500,000 cells/ml was added 20 µl of Pitstop 2 (5 mg/ml in DMSO) and 12 µl of Dynasore hydrate (5 mg/ml in DMSO). As a negative control, a 3 ml cell suspension at a cell density of 500,000 cells/ml was used to which was added 16 µl of filter-sterilized DMSO. The cell suspensions were transferred to a water bath on hotplate and were treated for 30 minutes at 37°C and 540 rpm. To the 6-ml cell suspension was then added 10 µl of doxorubicin (1 mg/ml). Two aliquots of 3 ml cell suspension were transferred to a reaction vessel in CEM (MW) and a

reaction vessel on hotplate (control). CEM microwave reaction vessel parameters were adjusted to 37°C, maximum power 10 W, and slow stirring (540 rpm). For control, the temperature of the water bath was pre-set to 37°C and the stirring speed of hotplate was set to 540 rpm. Samples were treated for 20 minutes under each condition. At the end of each treatment, two samples of 1 ml cell suspension were taken from each reaction vessel (MW and control) and were transferred to microcentrifuge tubes and spun at 2300 rcf for 3 min to obtain cell pellets. After the supernatant was removed by aspiration, the cell pellets were re-suspended in 1×PBS (1 ml) and were centrifuged for 3 min at 2300 rcf. The supernatant was discarded, and the cell pellet was re-suspended in 1 ml 1×PBS. The samples were analyzed by a BD Accuri C6 flow cytometer and the median fluorescence of each sample at FL-3 channel was recorded. Fluorescent images were obtained by a Carl Zeiss Axio Observer confocal microscope.

Human transferrin, Pitstop 2 and Dynasore hydrate assay

To a 6 ml cell suspension of MCF-7 cells at a density of 500,000 cells/ml was added 20 µl of Pitstop 2 (5 mg/ml in DMSO) and 12 µl of Dynasore hydrate (5 mg/ml in DMSO). As a negative control, a 6 ml cell suspension at a cell density of 500,000 cells/ml was used to which was added 32 µl of filter-sterilized DMSO. Each 6 ml cell suspension was divided to three aliquots of 2 ml in three reaction vessels. The 2-ml cell suspensions were transferred to a water bath on hotplate and were treated for 30 minutes at 37°C and 540 rpm. To each 2-ml cell suspension was then added 25 µl of Human transferrin 488A conjugate (Human Transferrin, CFTM488A, 1 mg/ml). The reaction vessels were incubated in the water bath for 20 minutes, the temperature of the water bath was kept at

37°C and the stirring speed of hotplate was kept at 540 rpm. At the end of the treatment, one sample of 1 ml cell suspension was taken from each reaction vessel and was transferred to a microcentrifuge tube and spun at 2300 rcf for 3 min to obtain cell pellets. After the supernatant was removed by aspiration, the cell pellets were re-suspended in 1×PBS (1 ml) and were centrifuged for 3 min at 2300 rcf. The supernatant was discarded, and the cell pellet was re-suspended in 1 ml 1×PBS. The samples were analyzed by a BD Accuri C6 flow cytometer and the median fluorescence of each sample at FL-1 channel was recorded. Fluorescent images were obtained by a Carl Zeiss Axio Observer confocal microscope.

Oligonucleotide uptake assay

To a 6 ml cell suspension at 500,000 cells/ml in modified DMEM was added 15 μl of FAM-labelled 18-nt oligodeoxynucleotide (FAM-5'-TTG GTG GAT AGT TAT TAG-3', FAM: fluorescein. IDT) at 1 mg/ml. Two aliquots of 3 ml cell suspension were transferred to the reaction vessel in CEM (for microwave treatment) and a reaction vessel in a water bath on hotplate (control treatment). As a negative control, a 3 ml cell suspension at a cell density of 500,000 cells/ml was used to which was added 7.5 μl of nuclease-free dH_2O.

CEM reaction vessel parameters were adjusted to 37°C, maximum power 10 W, and slow stirring (540 rpm). For control, the temperature of the water bath was pre-set to 37 °C and the stirring speed of hotplate was set to 540 rpm. Samples were treated for 20 minutes under each condition. At the end of each treatment, two samples of 1 ml cell suspension were taken from each reaction vessel (MW and control) and were transferred to

microcentrifuge tubes and spun at 2300 rcf for 3 min to obtain cell pellets. After the supernatant was removed by aspiration, the cell pellets were re-suspended in 1×PBS (1 ml) and centrifuged for 3 min at 2300 rcf. The supernatant was discarded, and the cell pellet was re-suspended in 1 ml 1×PBS. The samples were analyzed by a BD Accuri C6 flow cytometer and the median fluorescence of each sample in FL-1 channel (Em 533±15 nm) was recorded. Fluorescent images were obtained by a Carl Zeiss Axio Observer confocal microscope at EGFP (Ex. 470±20 nm, Em. 525±25 nm) channel.

Doxorubicin assay (Human fibroblast cells)

Cell density was measured by a hemocytometer. By dilution, cell density was adjusted to ~185,000 per ml prior to each experiment. To a 6 ml cell suspension in modified DMEM was added 10 µl of doxorubicin (1 mg/ml) to give a final concentration of 26 µM. Two aliquots of 3 ml cell suspension were transferred to the reaction vessel in CEM (for microwave treatment) and a reaction vessel in a water bath on hotplate (control treatment).

CEM reaction vessel parameters were adjusted to 37°C, maximum power 10 W, and slow stirring (540 rpm). For control, the temperature of the water bath was pre-set at 37°C and the stirring speed of hotplate was set to 540 rpm. Samples were treated for 20 minutes under each condition. At the end of each treatment, 2 samples of 1 ml were taken from each reaction vessel (MW and control) and were transferred to microcentrifuge tubes. The microcentrifuge tubes were spun at 2300 rcf for 3 min to obtain cell pellets. After aspirating the supernatant, the cell pellets were re-suspended in 1×PBS (1 ml) and were centrifuged for 3 min at 2300 rcf. The supernatant was discarded, and the cell pellet was

re-suspended in 0.75 ml 1×PBS. The samples were analyzed by a flow cytometer (BD Accuri) and the median fluorescence of each sample at FL-3 channel was recorded. Fluorescent images were obtained in channels mcherry (Ex: 560±20 nm, Em: 630±37.5 nm) and cy3 (Ex: 545±12.5 nm, Em: 605±35 nm).

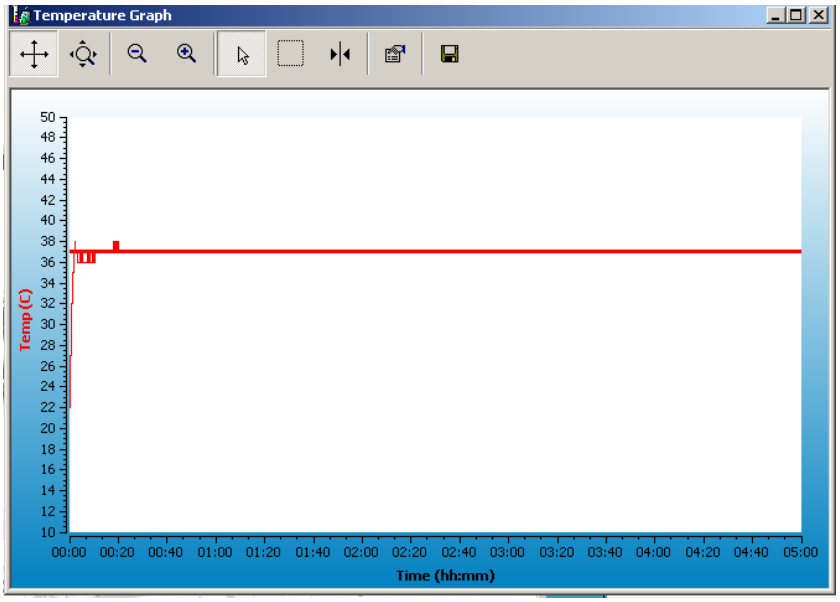
Media: Modified DMEM (Dulbecco's Modified Eagle Medium) contains 10% Fetal bovine serum, 2% non-essential amino acids, 1% penicillin/streptomycin.

Human fibroblast cell cultures: Human fibroblast (normal) cells were cultured in modified DMEM in 10 cm cell culture dishes (Sarstedt) until a confluent monolayer of adherent cells was obtained. The cells were washed twice with 1×PBS (4.0 ml, pH 7.4). Trypsin–EDTA solution (3 ml, 0.25%, Sigma, T4049) was then added and the plate was incubated at 37 °C for 4 min in a culture incubator (5% O_2, 5% CO_2, 37°C). DMEM (4.0 ml) was then added to the plate and the cell suspension was transferred to a 15 ml Falcon tube and centrifuged at 970 g for 3 min to obtain a cell pellet, which was used for subsequent experiments.

Statistical analysis

Student's t-Test (Two-Tailed, unequal variance) was carried out in Excel. Experiments with p values not greater than 0.05 are considered as statistically significant. In each graph presented in the results, a significant difference is identified between samples.

(a)



(b)

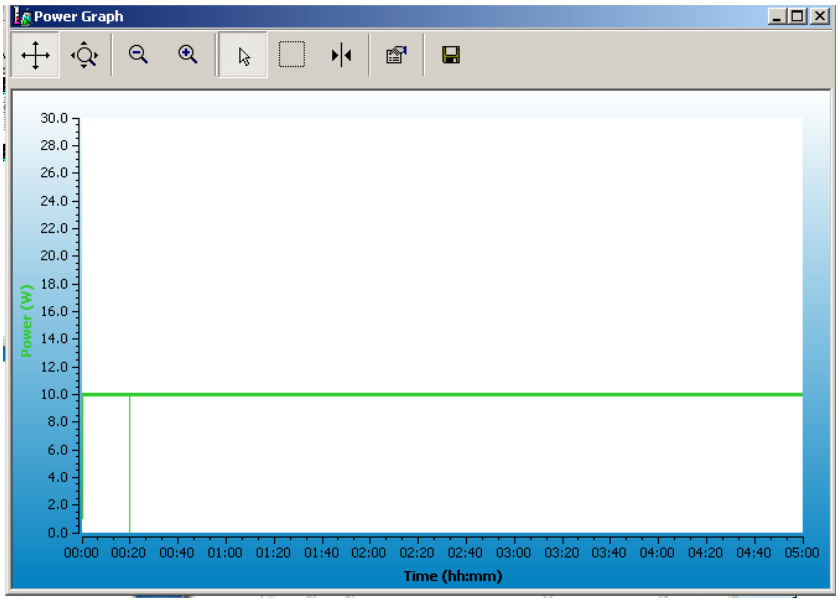


Figure 5.1S. (a). Representative temperature and (b) microwave power profiles in the microwave reactor.

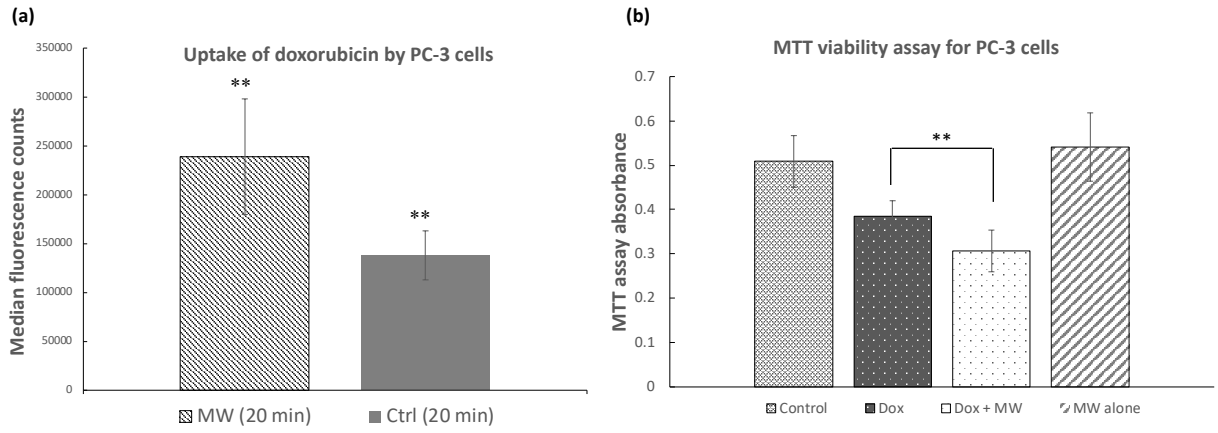


Figure 5.2S. (a). Microwave-assisted internalization of doxorubicin, and (b). MTT viability assays of PC-3 cells treated with doxorubicin and microwave. **P<0.05 for biological triplicate experiments. Error bars are shown as standard deviation. Student's t-Test (two-tailed) was performed for statistical analysis.

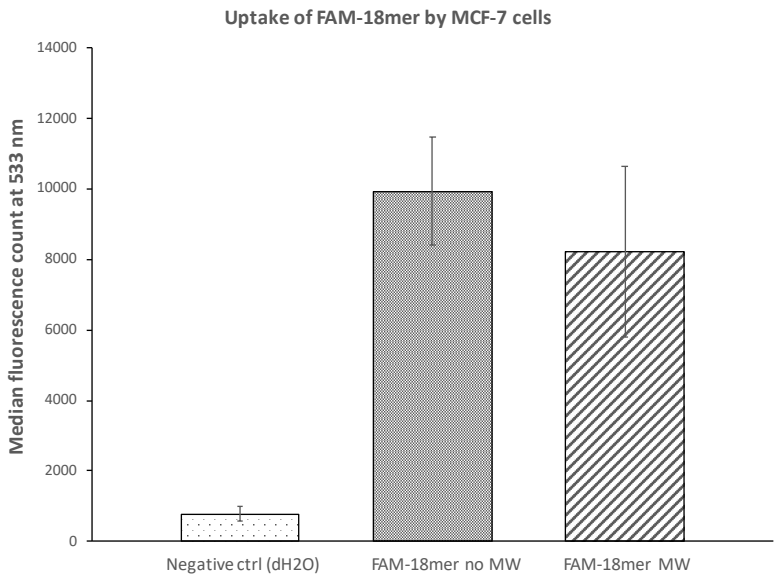


Figure 5.3S. Uptake of FAM-18mer oligonucleotide by MCF-7 cells. Cultures were carried out in biological triplicates. No oligonucleotide was added in the negative control cultures. FAM-18mer oligonucleotide was added in the other two cultures, one exposed to microwave, but not the other. Error bars are shown as standard deviation.

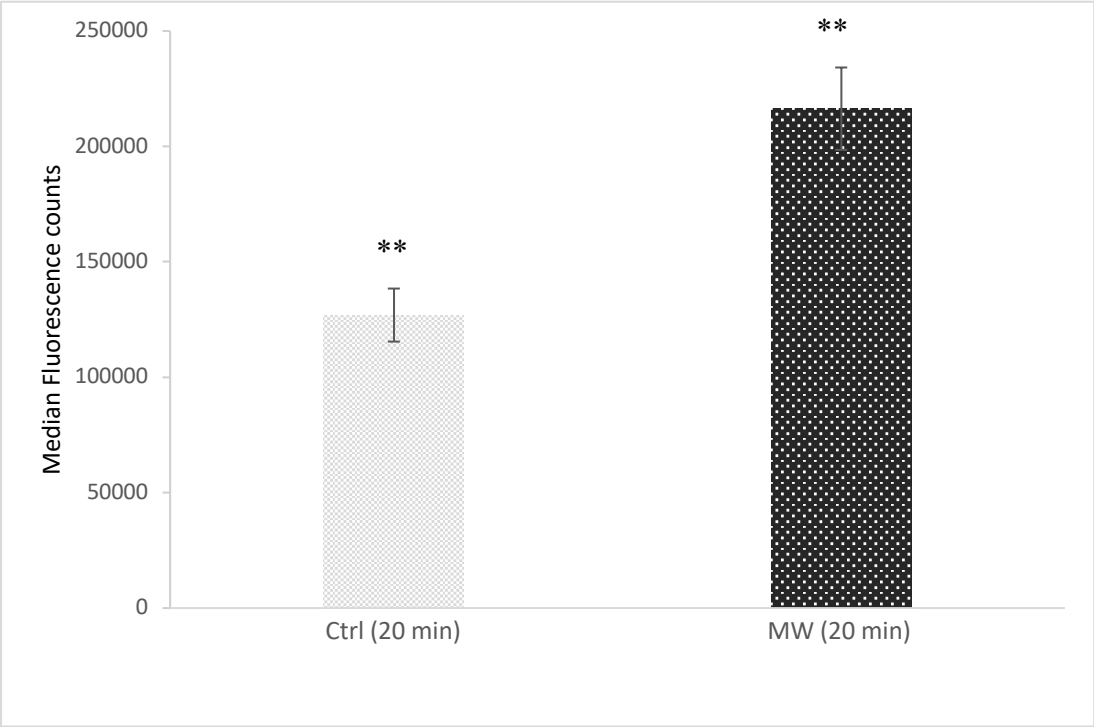


Figure 5.4S. Internalization of doxorubicin by human fibroblast cells treated with or without microwave. **P<0.05 for biological triplicate experiments. Error bars are shown as standard deviation. Student's t-Test (two-tailed) was performed for statistical analysis.

Chapter 6

Exposure to microwave irradiation at constant culture temperature slows the growth of *Escherichia coli* DE3 cells, leading to modified proteomic profiles

Introduction

With increased applications of electromagnetic waves in many aspects of our daily life, determining the effects of microwave irradiation on biological systems is not only essential, but far from fully elucidated. Notions of 'thermal' and 'non-thermal' effects (the latter is also referred to as 'microwave-specific effects') of microwaves have been under repeated scrutiny in the past few decades.[1-10] Some recent literature argued that due to the unique manner of energy transfer by microwaves, many observations that are unique to microwave exposure can be attributed to 'selective heating' of substances in a system of interest.[11,12] As a result, isolation of thermal from microwave-specific effects is challenging,[13-15] further complicating the interpretation of experimental results.

In this respect, we previously demonstrated that a CEM (Matthews, NC) microwave reactor operating at 2.45 GHz coupled with a Coolmate system allows for systems under investigation to remain typically within ±1 °C throughout experiments (with occasional brief fluctuations of ±2 °C), as measured in situ by a fibre optic temperature probe and an alcohol-based thermometer, when the system is subjected to low-power microwave irradiation while cooled simultaneously. We were particularly interested in studies in which relatively low microwave energy (up to 10 W) is used, as higher microwave output is, presumably, more likely to exacerbate the issue of local heating and generation of hotspots. With these considerations, we demonstrated that while the enzymatic activity of trypsin during digestion of substrates, such as casein, is increased by exposure to microwave irradiation (10 W) at constant bulk temperature,[16] a similar impact was not found for α-amylase and phosphatase.[17] We further demonstrated that exposure of PC-3

human prostate cancer cells to non-lethal microwave irradiation (10 W) at constant culture temperature (37 °C) likely led to perturbation of cell membrane properties, while no necrosis nor apoptosis was induced.[18] Most recently, we showed that uptake of the anticancer compound doxorubicin by PC-3 and human breast cancer MCF-7 cells was significantly enhanced when the cells were exposed to non-lethal microwave irradiation.[19]

In the present study, we extended our investigation to probe the influence of microwave exposure on bacterial growth while the culture temperature was maintained constant. While many reports show no differences in microorganisms exposed to microwave irradiation as compared with controls,[20-26] there is also literature showing that microorganisms respond differently to microwave irradiation of various frequencies, leading to altered growth rate or metabolic activities.[21,27-36] Comparisons of results from the literature are compounded by differences in at least the following factors: frequency and power output of the microwave used, duration and pattern of irradiation, choice of biological system, method used in temperature measurement, and the extent of temperature fluctuation.

This study focused on the exposure of *E. coli* DE3 cells to microwaves at 2.45 GHz, with relatively low power output (up to 10 W), while the culture temperatures were monitored *in situ* with a fibre optic temperature probe, maintaining the culture temperatures at 37 °C through simultaneous cooling. We report herein that *E. coli* growth is slowed under these

conditions. Furthermore, a top-down proteomic analysis of total proteins suggest that cellular metabolism is modified in bacteria exposed to microwave irradiation.

Results and discussions

Temperature control

In the present study, great care was taken to ensure that the temperature of the bacterial cultures was controlled through simultaneous cooling and constant stirring; cultures were kept sterile with the use of a foam plug (Figure 6.1). Pre-cooled fluid (Galden HT 110) was circulated through the jacket surrounding the culture tube in order to maintain culture temperatures. As we have previously established,[17] the temperatures measured by a fibre optic temperature probe and an alcohol-based thermometer are very consistent. For simplicity, a fibre optic temperature probe was used here, as it allowed us to readily keep track of culture temperatures in real-time using the Synergy program. As is shown in Figure 6.S1, temperatures of the culture were maintained at 37 °C (typically ±1 °C with occasional brief fluctuations of ±2 °C) throughout the 27.5-h growth period.

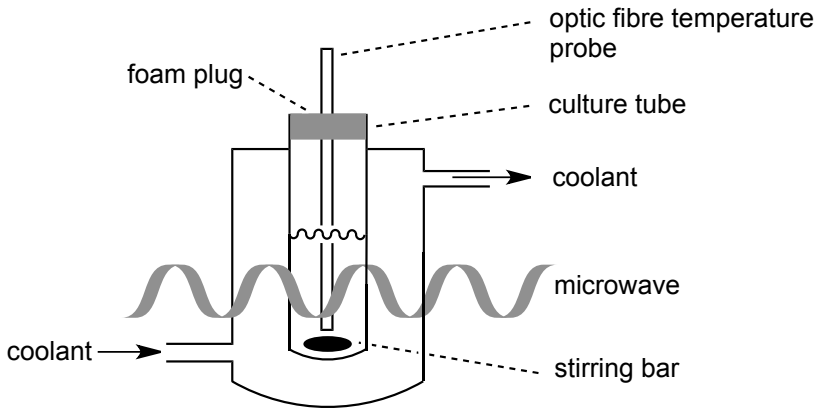


Figure 6.1. *E. coli* culture setup in a CEM microwave reactor.

It is worth noting that as the fibre optic temperature probe provides feedback to the microwave, the microwave power output from the magnetron was not constant. As shown in Figure 6.S2, while the maximal power output was set at 10 W, actual power output was below 10 W (>6 W for most of the irradiation period), but did not exceed this pre-set maximal value.

E. coli growth curve

As shown in Figure 6.2, the growth rates of *E. coli,* both in the presence and absence of microwave exposure, were slower than that in a shaking incubator, which is the result of less efficient oxygen transfer in the culture tubes under stirring compared with cultures in a shaking incubator. The growth of *E. coli* in the presence of maximal 10 W microwave irradiation at 37 °C was found to be significantly slower than control cultures not exposed to microwave irradiation.

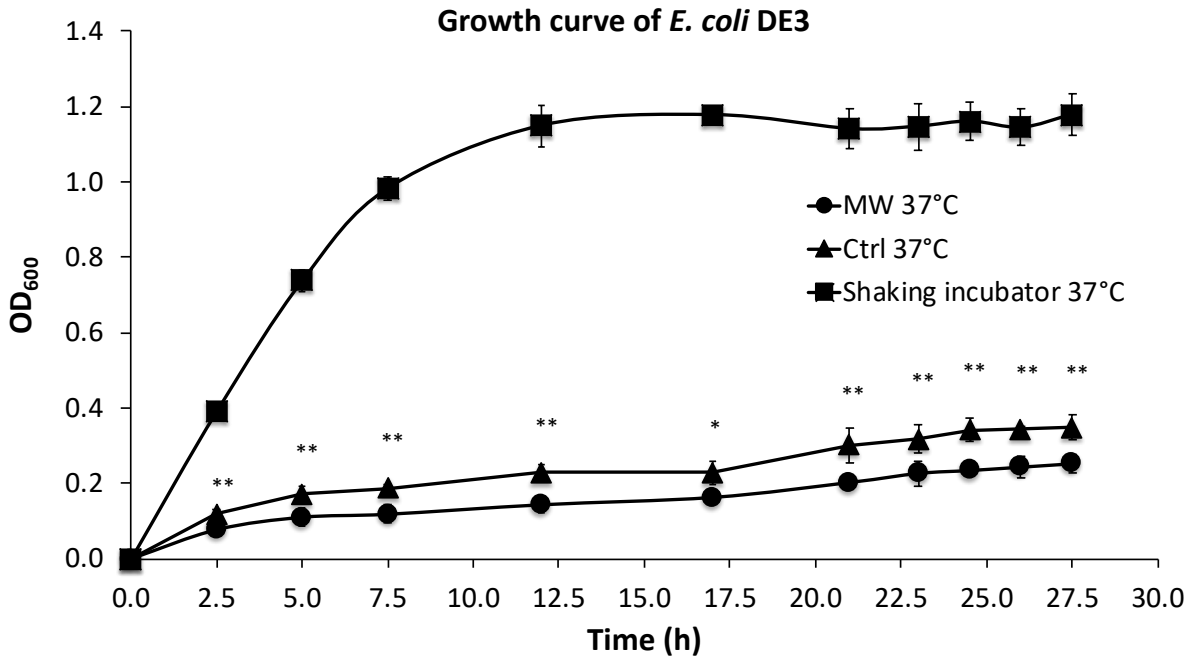


Figure 6.2. Growth curves of *E. coli.* ■: cultured at 37 °C in a shaking incubator at 260 rpm; •: cultured with stirring at 37 °C in a tube in the presence of microwave irradiation; ▲: cultured with stirring in a tube at 37 °C in an oil-bath in the absence of microwave exposure. All experiments were an average of biological triplicates. (Student's t-Test, Two-tailed); *: $P<0.05$; **: $P<0.01$ calculated for OD_{600} of cultures treated with microwave and control in oil bath. Graph shows mean ± std. dev.

Dissolved oxygen (DO) level in tube cultures

Considering the possibility that the slower growth of *E. coli* in the presence of microwave irradiation might be due to lower DO concentration when cultures are exposed to microwave irradiation, experiments were carried out to compare the DO levels in tube cultures isolated from the environment with foam plugs, in the presence and absence (control) of microwave irradiation.

Table 6.S1 shows that the DO levels are slightly lower in the LB media treated with microwave irradiation relative to the control. This observation is consistent with a recent study that demonstrated efficient degassing by microwave exposure, however, at much higher temperatures.[37]

Recovery of growth in the *E. coli* culture exposed to microwave irradiation

We were also interested in exploring whether the slower growth of *E. coli* subjected to microwave irradiation is reversible when microwave exposure stops. To this end, after *E. coli* cultures were exposed to maximal 10 W microwave irradiation at 37 °C for 5 h, the cultures were then incubated at 37 °C in an oil-bath, and growth rate (measured by OD_{600}) was assessed. As depicted in Figure 6.3, the difference in cell density between the microwave-treated and control cultures decreased when microwave irradiation was stopped. Viability tests revealed that 21 h after microwave exposure ceased, the difference in viable cell counts in both cultures was not significant (Figure 6.4).

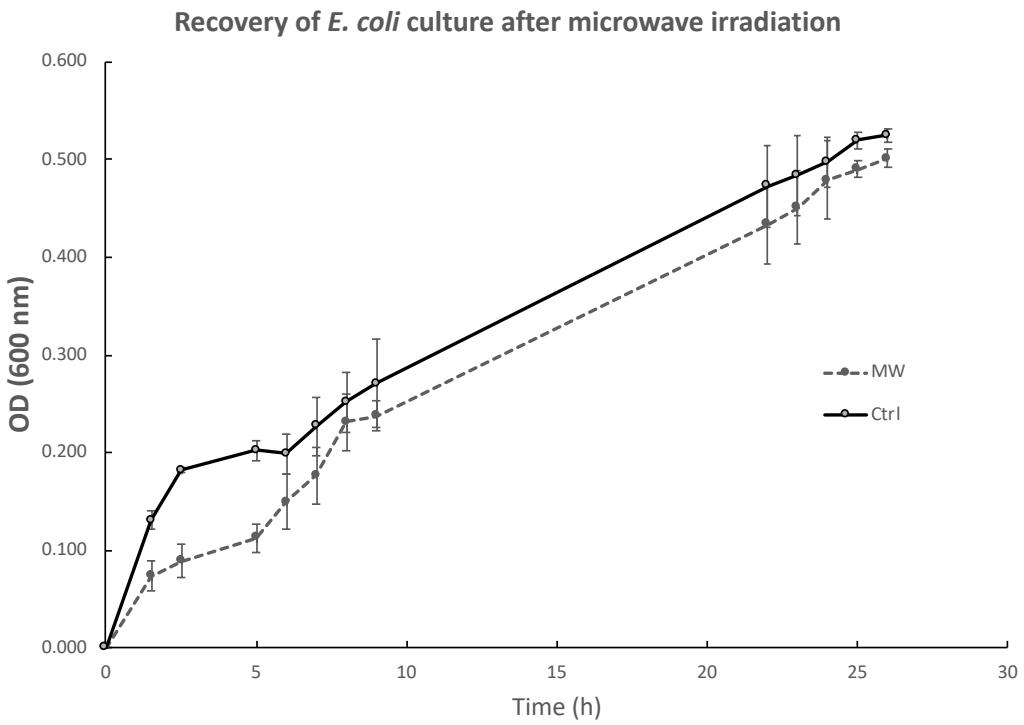


Figure 6.3. Recovery of *E. coli* growth after ceasing microwave exposure at 5 h as indicated by the decreased difference in OD_{600}. Both control and microwave-treated cultures were carried out in biological triplicates. Graph shows mean ± std. dev.

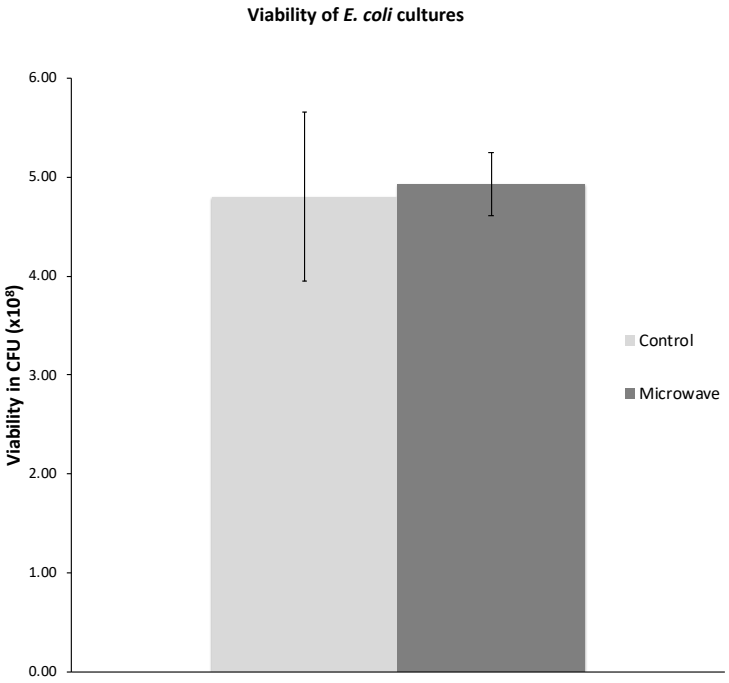


Figure 6.4. Viable cell counts 21 h after microwave exposure as compared with control. Graph shows mean ± std. dev.

Two-dimensional gel electrophoresis (2DE) of total proteins isolated from *E. coli* DE3 cultures

Studies have shown that physical phenomenon such as electromagnetic waves can act as stimuli that may trigger responses in biological systems.[38,39] Based on the current observations that *E. coli* cells grew more slowly in the presence of microwave irradiation, we became interested in studying the protein profiles, which could provide insight into potentially altered biological processes in *E. coli* under microwave exposure.

In order to investigate this scenario, total proteins were isolated from both control and microwave-treated *E. coli* cultures for 2DE-based proteomic analysis. Proteins from three biological replicates from both control and microwave-treated *E. coli* cultures were extracted and analyzed by 2DE in technical triplicates (all gel images are available in Figure 6.S3). Changes in spot densities between the resolved proteomes of microwave-treated vs. control *E. coli* were identified by quantitative image analysis. Here, 10 spots were identified as changing significantly, with three decreasing and seven increasing in abundance (Figure 6.5).

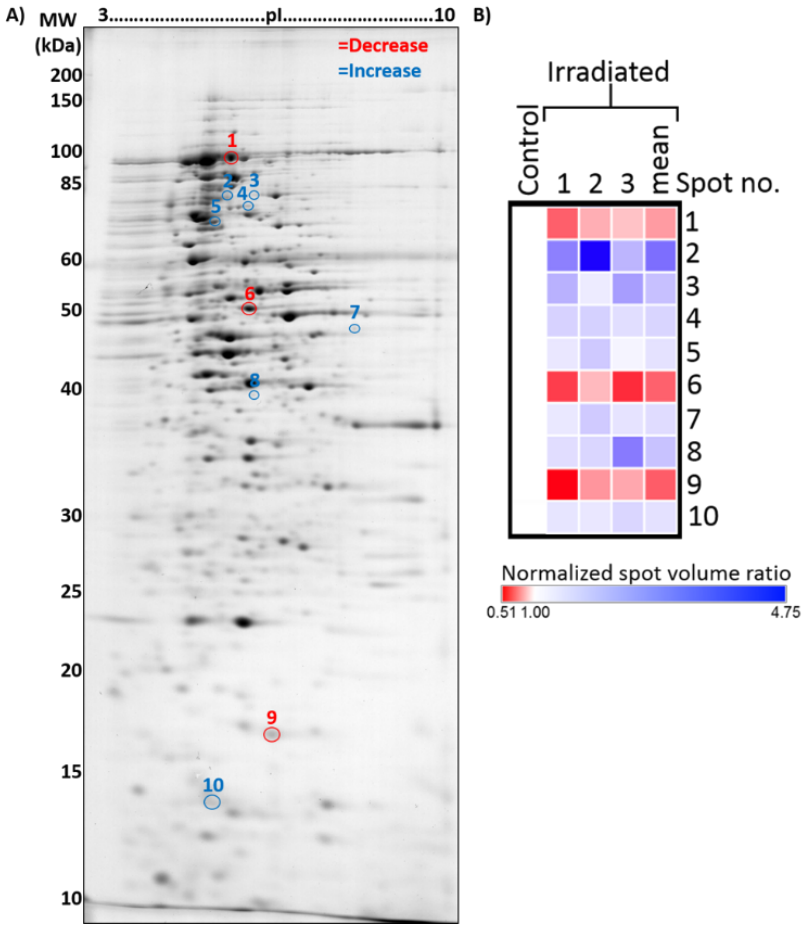


Figure 6.5. Representative 2DE gel used for the analysis of proteins extracted from control and microwave-treated *E. coli* cultures. A) Annotated 2DE gel image showing protein spots which changed in abundance following microwave irradiation. Spots which decreased in abundance are delineated in red; those which increased are delineated in blue. Refer to Table 6.1 for calibrated pI and molecular weights of each delineated spot. B) Heat map displaying normalised spot volume ratios in irradiated cultures relative to control cultures, across three biological replicates, averaged from three technical gel replicates each. Mean ratio is also shown.

Mass spectrometric (MS) analysis

Using the criteria described in the experimental section, within these 10 resolved spots a total of 42 proteins were identified by comparing the MS data against known *E. coli* K12 proteins (Table 6.1). These proteins were grouped into two clusters according to the spot intensity seen in the 2D gels, one containing 16 proteins with overall decreased abundance and another containing 26 proteins with overall increased abundance in the microwave-treated cultures, respectively. Figure 6.S4 shows the LC-MS chromatograms of the 10 resolved protein spots.

Table 6.1. Summary of the 42 proteins identified by mass spectrometry. The molecular weight (MW) and isoelectric point (pI) values determined by 2DE vs. theoretical are presented for comparison.

Protein spot	Abundance in irradiated *E. coli* [fold-change (p-value*)]	Protein ID	Accession	Coverage (%)	No. of unique peptides	Theoretical MW[kDa]/pI	Calculated** MW[kDa]/pI	Sequest score
1	↓1.3 (0.004)	Aconitate hydratase B	P36683	44	38	93.4/5.4	83.4/4.9	846.66
		Pyruvate dehydrogenase E1 component	P0AFG8	25	22	99.6/5.7		85.02
		Aldehyde-alcohol dehydrogenase	P0A9Q7	17	12	96.1/6.8		71.4
		2-oxoglutarate dehydrogenase E1 component	P0AFG3	12	9	105/6.5		23.09
		NADH-quinone oxidoreductase subunit G	P33602	9	9	100.2/6.3		22.31
		Leucine-tRNA ligase	P07813	9	7	97.2/5.3		17.1
		Elongation factor G	P0A6M8	8	4	77.5/5.4		9.61
		DNA mismatch repair protein MutS	P23909	3	2	95.2/5.55		4.3
		DNA gyrase subunit A	P0AES4	2	2	96.9/5.2		4.22
2	↑3.0 (<0.001)	GTP-binding protein TypA/BipA	P32132	13	6	67.3/5.3	73.4/4.8	5.33
		Catalase-peroxidase	P13029	8	5	80/5.3		5.31
		Formate acetyltransferase 1	P09373	4	3	85.3/6.0		6.01
		Polyribonucleotide nucleotidyltransferase	P05055	3	2	77.1/5.2		5.21
3	↑2.0 (<0.001)	Formate acetyltransferase 1	P09373	16	11	85.3/6.0	72.7/5.3	6.01
		Phosphoenolpyruvate-dependent phosphotransferase system	P37177	3	2	83.7/5.8		5.78
4	↑1.6 (<0.001)	Exoribonuclease 2	P30850	14	7	72.4/5.6	70.6/5.2	5.62
		Chaperone protein ClpB	P63284	7	5	95.5/5.5		5.52
		Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	P06959	5	3	66.1/5.2		5.17
		Glycine-tRNA ligase beta subunit	P00961	2	2	76.8/5.4		5.44
		Bifunctional polymyxin resistance protein ArnA	P77398	3	2	74.2/6.9		6.87
5	↑1.4 (0.001)	Chaperone protein HtpG	P0A6Z3	49	33	71.4/5.2	67.7/4.7	5.21
		Lysine-tRNA ligase, heat inducible	P0A8N5	26	12	57.8/5.2		5.24
		Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	P06959	14	7	66.1/5.2		5.17
		Proline-tRNA ligase	P16659	8	4	63.7/5.2		5.24
		30S ribosomal protein S1	P0AG67	6	4	61.1/5.0		4.98
		Polyribonucleotide nucleotidyltransferase	P05055	3	2	77.1/5.2		5.21
		Methionine-tRNA ligase	P00959	3	2	76.2/5.9		5.86

6	↓1.5 (<0.001)	D-tagatose-1,6-bisphosphate aldolase subunit GatZ	P0C8J8	18	7	47.1/5.8	53.7/5.2	5.77
		Periplasmic pH-dependent serine endoprotease DegQ	P39099	8	3	47.2/6.0		5.95
		Dihydrofolate synthase/folylpolyglutamate synthase	P08192	5	2	45.4/5.8		5.8
		Xaa-Pro aminopeptidase	P15034	6	2	49.8/5.4		5.39
		Bifunctional protein HldE	P76658	5	2	51.0/5.4		5.41
7	↑1.5 (0.006)	NADH-quinone oxidoreductase subunit F	P31979	12	5	49.3/6.9	49.9/6.6	6.86
8	↑1.9 (<0.001)	Aspartate aminotransferase	P00509	17	7	43.5/5.8	43.3/5.3	5.77
		NADPH-dependent curcumin reductase	P76113	7	2	37.6/5.8		5.82
		Aminomethyltransferase	P27248	5	2	40.1/5.6		5.57
		Maltose-binding periplasmic protein	P0AEX9	6	2	43.4/5.7		5.71
		Fructose-bisphosphate aldolase class 2	P0AB71	6	2	39.1/5.9		5.86
9	↓1.5 (0.006)	DNA protection during starvation protein	P0ABT2	50	12	18.7/6.1	16.9/5.6	6.11
		50S ribosomal protein L13	P0AA10	10	2	16.0/9.9		9.91
10	↑1.4 (0.011)	10 kDa chaperonin	P0A6F9	65	6	10.4/5.2	13.4/4.9	5.24
		30S ribosomal protein S6	P02358	13	2	15.7/5.0		5

*Significantly different from control, as displayed in the Delta2D quantitation table (Student's t-Test); **Calculated in Delta2D, calibrated with Bio-Rad 2-D PAGE standards.

Analysis of the proteins with decreased levels revealed that many proteins are involved in metabolic pathways, including the citric acid cycle, such as aconitate hydratase B, pyruvate dehydrogenase E1 component, aldehyde-alcohol dehydrogenase, 2-oxoglutarate dehydrogenase E1 component, NADH-quinone oxidoreductase subunit G, D-tagatose-1,6-bisphosphate aldolase subunit, periplasmic pH-dependent serine endoprotease, dihydrofolate synthase/folylpolyglutamate synthase and bifunctional protein HldE.

For the 26 proteins with increased levels, four enzymes required for the aminoacyl-tRNA biosynthesis pathway were identified, glycine-tRNA ligase (beta subunit), lysine-tRNA ligase (heat inducible), proline-tRNA ligase and methionine-tRNA ligase.

These results suggest that exposure of *E. coli* to microwave irradiation under the experimental conditions led to slower growth, likely due to stress imposed by microwave exposure. In response to the stimulation, metabolism of *E. coli* cells is down-regulated. The finding that four enzymes involved in tRNA synthesis were up-regulated also corroborates this hypothesis, as the literature points to the regulation of cellular tRNA levels as a response to stresses, such as starvation and heat.[40-42]

Taken together, the proteomic results from this study support the possibility that exposure of *E. coli* to non-lethal microwave irradiation at constant culture temperatures can be perceived by the microorganism as a stimulus. In adaptation, the bacteria modulate their metabolism and tRNA biosynthesis.

Materials and methods

Chemicals were purchased from Sigma-Aldrich unless otherwise stated, and were used without further purification. *E. coli* DE3 was a gift from Professor Charles Dépres' lab (Department of Biological Sciences, Brock University).

Microwave irradiation

A CEM Discovery Coolmate microwave system that operates at 2.45 GHz was used for microwave irradiation, and controlled with Synergy software from CEM (Matthews, NC). The standard Reaction Vessel (CEM part number 168302) was used as culture tubes, inserted into the standard Attenuator Assembly (CEM part number 542476), which provides cooling to the culture through circulating coolant. Solvay Solexis H Galden Coolant Zt 130 was pre-cooled in the coolant reservoir of Coolmate by liquid nitrogen. Temperature of cultures was preset as desired in Synergy and maintained through adjustment of the temperature of coolant and flow rates. The instrument and culture set-up are described in Figure 6.1. Speed of the stirring bar was determined with an Omega HHT13 Tachometer.

Generation of *E. coli* growth curves from cultures in a shaking incubator

Single bacterial colonies from LB agar plates were grown in LB broth in a shaking incubator at 37 °C and 260 rpm until an OD_{600} in a range of 0.8–0.9 was reached. Aliquots (200 µl) were taken at appropriate intervals, and absorbance was read at 600 nm using a Biotek 405U microtitre plate reader.

Generation of *E. coli* growth curves from tube cultures in the presence of microwave and in hotplate oil-bath

100 µl of cultures grown in the shaking incubator was added to fresh LB media (4.9 ml, to make a 1:50 dilution) in the reaction vessel in the CEM microwave reactor and exposed to a maximal 10 W microwave irradiation while the culture temperature was maintained at 37 °C through simultaneous cooling with constant stirring (stirring speed set as "high" at 800±20 rpm). The speed of the stirring bar was measured in rpm by an Omega HHT13-Kit tachometer. The culture tube was kept sterile by a foam plug. Aliquots (200 µl) were taken at appropriate intervals, and absorbance was read at 600 nm using a Biotek 405U microtitre plate reader.

For growth recovery experiments, microwave irradiation was terminated after 5 h and the culture tube was transferred to an oil-bath and incubated at 37 °C. Growth was monitored for 26 h after initial inoculation by taking aliquots (200 µl) for OD_{600} measurement. A total viable cell count test was carried out at the end of each experiment.

As a control, *E. coli* cultures were incubated in an oil-bath at 37 °C and stirred. The speed of the stirring bar was measured by a Tachometer to be 800±20 rpm. Culture growth was monitored for 26 h after inoculation. A total viable cell count test as described below was carried out at the end of each experiment.

Total viable cell counts

To determine the total viable cell count (colony-forming units, CFU, per ml) at the end of each growth curve experiment, bacterial cultures were diluted up to 10^8-fold by serial dilution. Aliquots (100 µl) of each dilution were streaked on an LB agar plate. Single colonies were counted after 18 h of incubation at 37 °C in a static incubator.

DO measurements

The apparatus used for measuring DO levels was made in-house (Figure 6.6). Instead of an external cooling jacket, coolant was circulated through an internal cooling tube inserted into the culture tube that contained uninoculated LB media used in the growth experiments, isolated from the environment by a foam plug. The temperature inside the tube was monitored by a fibre optic temperature probe.

Statistical analysis

Student's t-Test (Two-Tailed) was carried out in Excel. Experiments with p values not greater than 0.05 are considered as statistically significant. In each graph presented in the results, a significant difference is identified between samples.

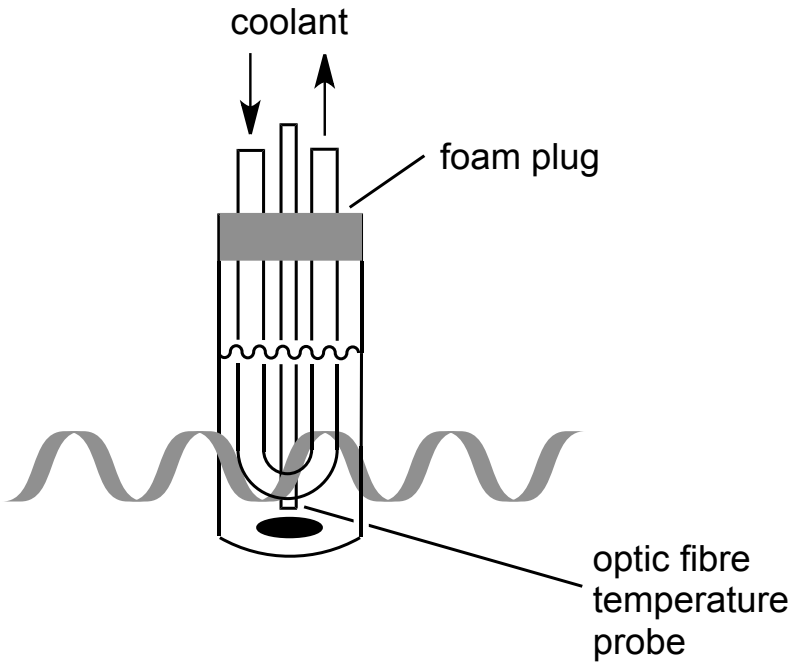


Figure 6.6. Setup for DO measurement. LB media isolated from the environment with the use of a foam plug were cooled through circulation in an internal cooling tube.

LB media (35 ml) was irradiated with microwaves as described above, with stirring. At 1 and 2 h, the foam plug and cooling coil were removed, the vessel was quickly sealed, and DO was measured by inserting a Vernier Micro SD Optical Dissolved Oxygen probe into the vessel. DO levels were recorded after values stabilized (usually after two minutes). DO levels in non-irradiated controls were measured accordingly.

Bacterial culture and total protein extraction

LB media was inoculated with single colonies of *E. coli* DE3 and incubated in a shaking incubator at 37 °C and 260 rpm until an OD_{600} in a range of 1.32–1.36 was reached. A

portion of this pre-culture (4.5 ml) was then added to fresh LB media (40.5 ml, to make a 1:10 dilution) in the reaction vessel in CEM microwave reactor and exposed to a maximal 10 W microwave irradiation while the culture temperature was maintained at 37 °C through simultaneous cooling and stirring was set at high. The speed of the stirring bar was measured by a Tachometer (Omega HHT13-Kit) to be 800±20 rpm. The control was prepared similarly, except that the reaction vessel was placed in a water bath on a hotplate at 37 °C. The reaction vessels were isolated from the environment by foam plugs. After 5 h, cultures were transferred to 50 ml-Falcon tubes and centrifuged at 4255 RCF for 20 minutes at 4 °C. The supernatants were discarded and the cell pellets were washed three times by resuspension in 1xPBS (30 ml), and centrifugated as above. Supernatant-free cell pellets were flash frozen with liquid nitrogen and were stored at -80 °C. Three biological replicates per condition were prepared for analysis.

Pellets were homogenized via automated frozen disruption and solubilized in protein/kinase/phosphatase inhibitor-supplemented 2DE lysis buffer as described previously.[43] Sample aliquots were snap-frozen and stored at -80 °C. Total protein concentrations were determined using a solid-phase dot-blot assay as described previously,[44] with BSA as the calibration standard.

2DE and in-gel protein detection

All methods for immobilized pH gradient strip (IPG) rehydration, isoelectric focusing (IEF), and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described in detail previously.[45] Briefly, 100 µg total protein from each sample was reduced and alkylated prior to passive rehydration into a 7 cm pH

3-10 non-linear (ReadyStrip™ IPG Strip (Bio-Rad, Hercules, CA)) for 16 h. IEF in a Protean i12 IEF Cell (Bio-Rad, Hercules, CA) was at 4000 V for 37500 VH with several electrode wick changes during linear voltage ramping to facilitate desalting. Focused IPGs were equilibrated with reduction and alkylation prior to further protein resolution in large-format (18 x 17 x 0.1 cm) 12.5% T gels. Two IPGs were adjacently resolved per large gel, yielding 'mini-tall' gels (i.e. 7 cm first-dimension and 18 cm long second-dimension). Unstained protein marker (10-200 kDa; New England Biolabs, Ipswich, MA) were resolved in parallel to approximate resolved proteoform molecular weights. Electrophoresis was carried out in a cold room at 4 °C, for 20 min at 300 V then 120 V to completion (~18 h). Gels were fixed and stained using highly sensitive colloidal Coomassie Brilliant Blue (cCBB) as described previously.[46] Three technical replicates per biological replicate were assessed.

Gel imaging and analysis

Destained gels were imaged via transmissive densitometry using the GS-900™ Calibrated Densitometer (Bio-Rad, Hercules) at maximal scanning resolution. 16-Bit greyscale TIFF images were exported for analysis. Quantitative image analysis was performed using Delta2D (Decodon GmbH, Greifswald, Germany) following the manufacturer-recommended workflow (image alignment through to statistical analysis), with automated processes (i.e. warping, consensus spot pattern) manually refined to ensure accuracy.[47]

A Welch's t-Test was carried out for all protein spots across the control and treatment groups using the following parameters: p-value based on all permutations, critical p-value

of 0.01, and false discovery rate from permutations to exceed no more than 5 proteins. From the resulting list of significantly altered spots (18), any spot with a relative standard deviation (RSD) of >40% of the normalized volume in either experimental group was excluded. The remaining spots, the majority of which exhibited ≤30% normalised volume RSD across all replicates, were considered genuine after again assessing quality by manual comparison to ensure the highest possible reliability.

For isoelectric point (pI) and molecular weight (kDa) calibration, one mini-tall gel in which 2-D PAGE standards (Bio-Rad, Hercules, CA) were resolved, and one mini-tall gel in which 10 µg *E. coli* sample spiked with 2-D PAGE standards was resolved (to ensure correct alignment) were prepared as described above. Calibration gel images were imported, warped, and annotated in Delta2D according to manufacturer recommendations for use of the in-built calibration tool.

Spot picking and in-gel digestion

Protein spots of interest were manually excised from several gels from both experimental groups, pooled, and transferred to microcentrifuge tubes. In-gel tryptic digestion was carried out as described previously[47] using 3 ng/µl trypsin with digestion performed at 4 °C for 30 min followed by 12 h at ambient temperature. Peptide solutions were recovered into microcentrifuge tubes and dried in a speed vacuum. Samples were shipped at ambient temperature for LC-MS/MS analysis.

Mass spectrometry analysis and protein identification

Mass spectrometry analysis was carried out using a Q-Exactive mass spectrometer. Data-dependant acquisition was performed on the top 10 peptides per duty cycle with automatic switching between MS and MS/MS. Full-scan MS mode (375–1600 m/z) was operated at a resolution of 70,000 with automatic gain control (AGC) and a target of 1×10^6 ions. Ions selected for MS/MS (fixed first mass 100 m/z) were subjected to the following parameters: resolution 17,500, target of 1×10^5 ions, 1.5 m/z isolation window, normalized collision energy 27.0 V and dynamic exclusion 20.0 s. Source ionization parameters were as follows: spray voltage, 1.9 kV; capillary temperature, 280 °C; and s-lens RF level, 50.0. Chromatographic separation of peptides was accomplished using a Zorbax 300SB-C18 column (3.5 µm i.d. x 150 mm, particle size 5 µm, pore size 100 Å, Agilent Technologies, Wilminton, DE). Here, peptides were loaded onto a Zorbax 300SB-C18 trap cartridge at a flow rate of 2 µl per minute for 10 min. After washing with 0.1% formic acid the peptides were eluted using a 5–40% B gradient for 30 min at a flow rate of 250 nl min^{-1} acid (mobile phase A = 0.1% formic acid; mobile phase B = 0.1% formic acid in acetonitrile) on an Agilent 1260 capillary/nano system.

LC-MS/MS results were searched using Proteome Discoverer (version 2.2, Thermo Scientific against the Uniprot *E. coli* (strain K12) (4,340 entries)) and the Proteome Discoverer contaminants database in which raw files were searched using the Sequest HT algorithm. Peptides produced by digestion with trypsin, with a maximum of two missed cleavages, were matched using precursor and fragment mass tolerances of 10 ppm and 0.04 Da, respectively. Carbamidomethylation of cysteine residues (C) was selected as a static modification. Oxidation (M), deamidation (NQ) and acetyl (protein N-term) were

chosen as dynamic modifications. Peptide spectrum matches (PSMs) were verified based on q-values set to 1% false discovery rate (FDR).

Conclusions

When *E. coli* cultures were exposed to up to 10 W microwave irradiation, while temperature was maintained at 37±1 °C with occasional brief fluctuations of ±2 °C, the growth slowed relative to non-irradiated cultures. When microwave irradiation was terminated, however, growth was restored. The identifications of the altered proteins suggest that the microwave exposure likely leads to the alteration of the proteomic profiles of the bacteria, however the long-term effects of such exposure on bacterial physiology remain unclear at this time. In connection with our previous findings, it appears that exposure of biological systems to non-lethal microwave irradiation can lead to alterations of enzymatic activities, membrane properties, and cellular metabolism. Future work will focus on the validation of microwave exposure related biological processes in *E. coli* and transcriptomic analysis of the proteins involved in these processes.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Keywords

Microwave specific effect • bacterial growth • 2D-gel electrophoresis • mass spectrometry • Top-down Proteomics • pathway analysis

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Supplementary material

Exposure to microwave irradiation at constant culture temperature slows the growth of *Escherichia coli* DE3 cells, leading to modified proteomic profiles

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Note: All experiments other than 2DE and mass spectrometry were performed by Sina Atrin Mazinani (Bacterial culture and total protein extraction was performed by Sina Atrin Mazinani and Nour Noaman)**.** Nour Noaman performed 2DE and in-gel protein detection, gel imaging and analysis and spot picking and in-gel digestion. Melissa Pergande performed mass spectrometry analysis and protein identification.

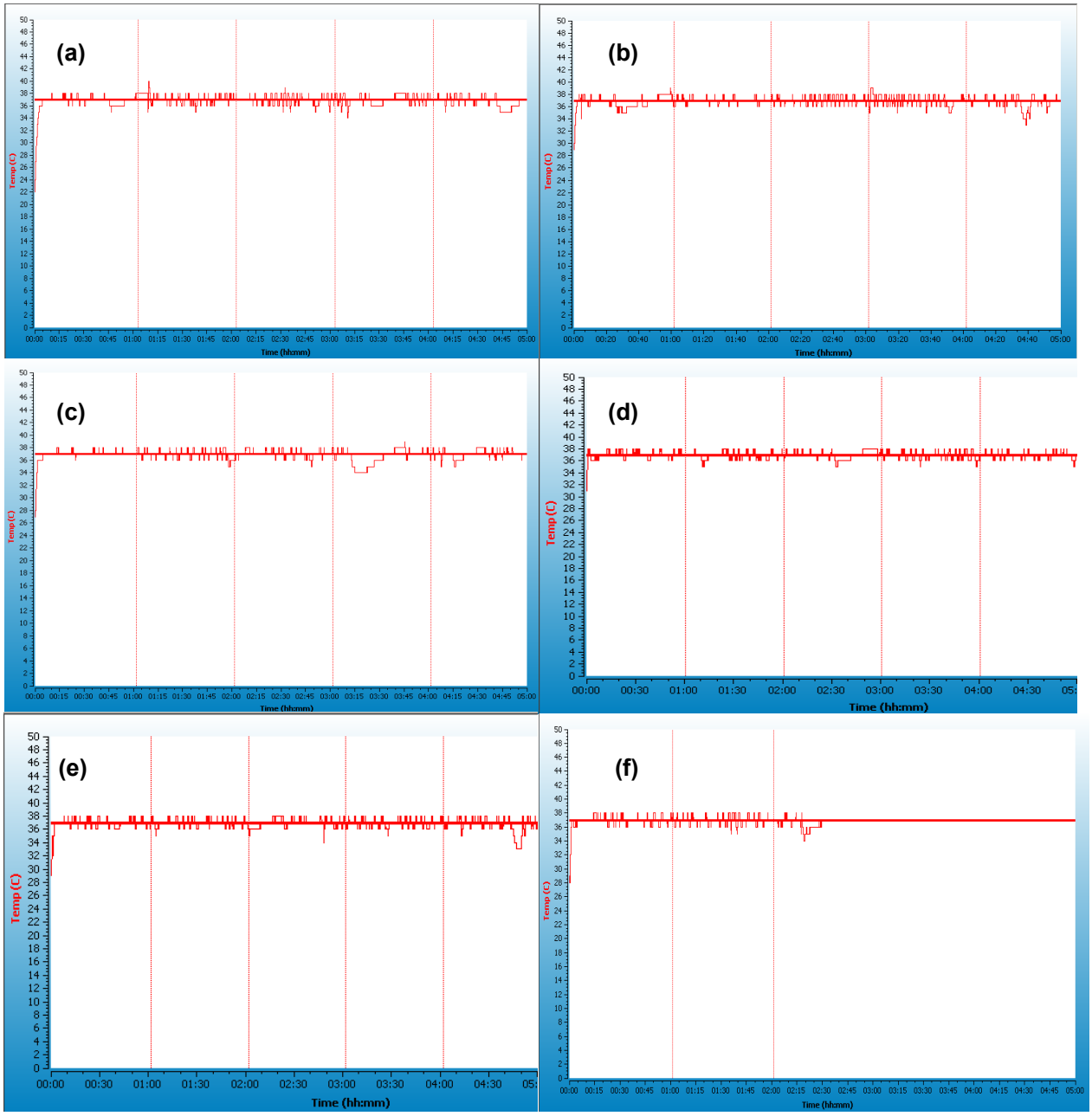


Figure 6.S1. Temperature profiles of cultures throughout the growth period (total 27.5 hours). Note: as the Synergy software keeps track of temperature profiles on five-hour frames, multiple frames (a-f) were provided to cover the entire growth period.

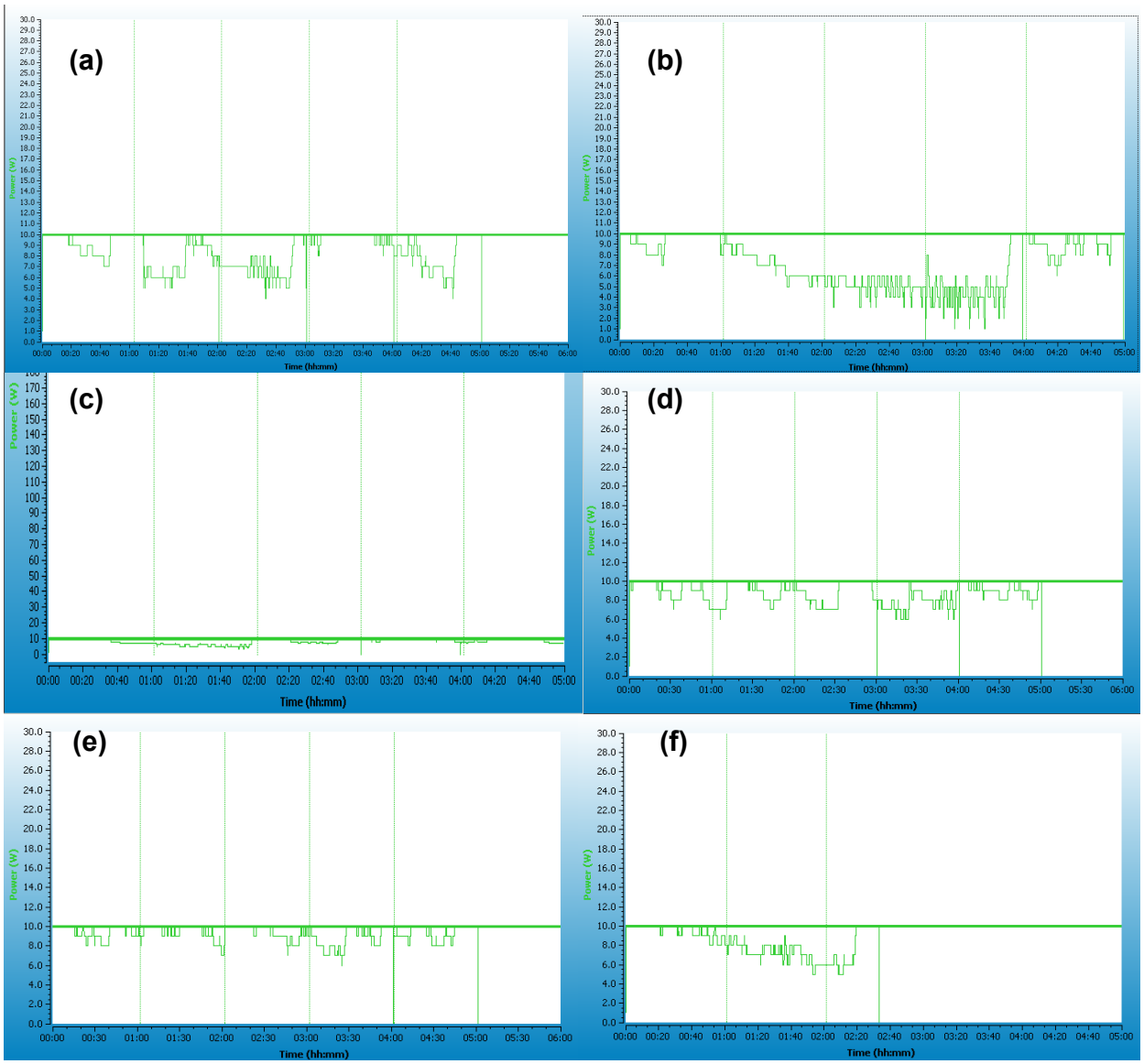


Figure 6.S2. Profiles of microwave power output throughout the growth period (total 27.5 hours). Note: as the Synergy software only keeps track of power output profiles on five-hour frames, multiple frames (a-f) were provided to cover the entire growth period.

Table 6.1S. DO levels in LB media.

	DO concentration (mg/L) average of two replicate experiments	
	1 hour	2 hour
Control	7.19 (7.23/7.15)	6.98 (6.94/7.01)
Microwave exposed	6.50 (6.46/6.55)	6.40 (6.37/6.44)

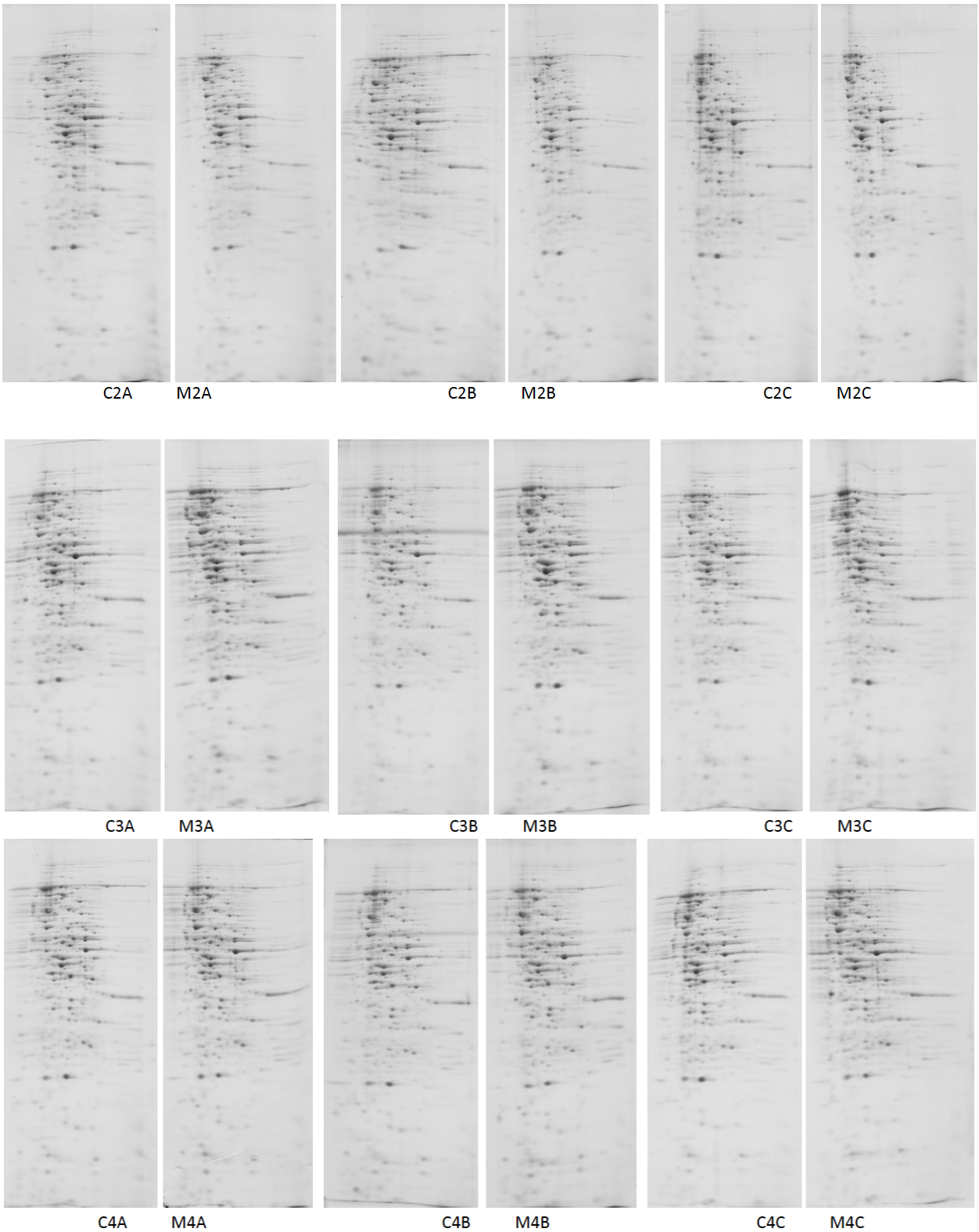
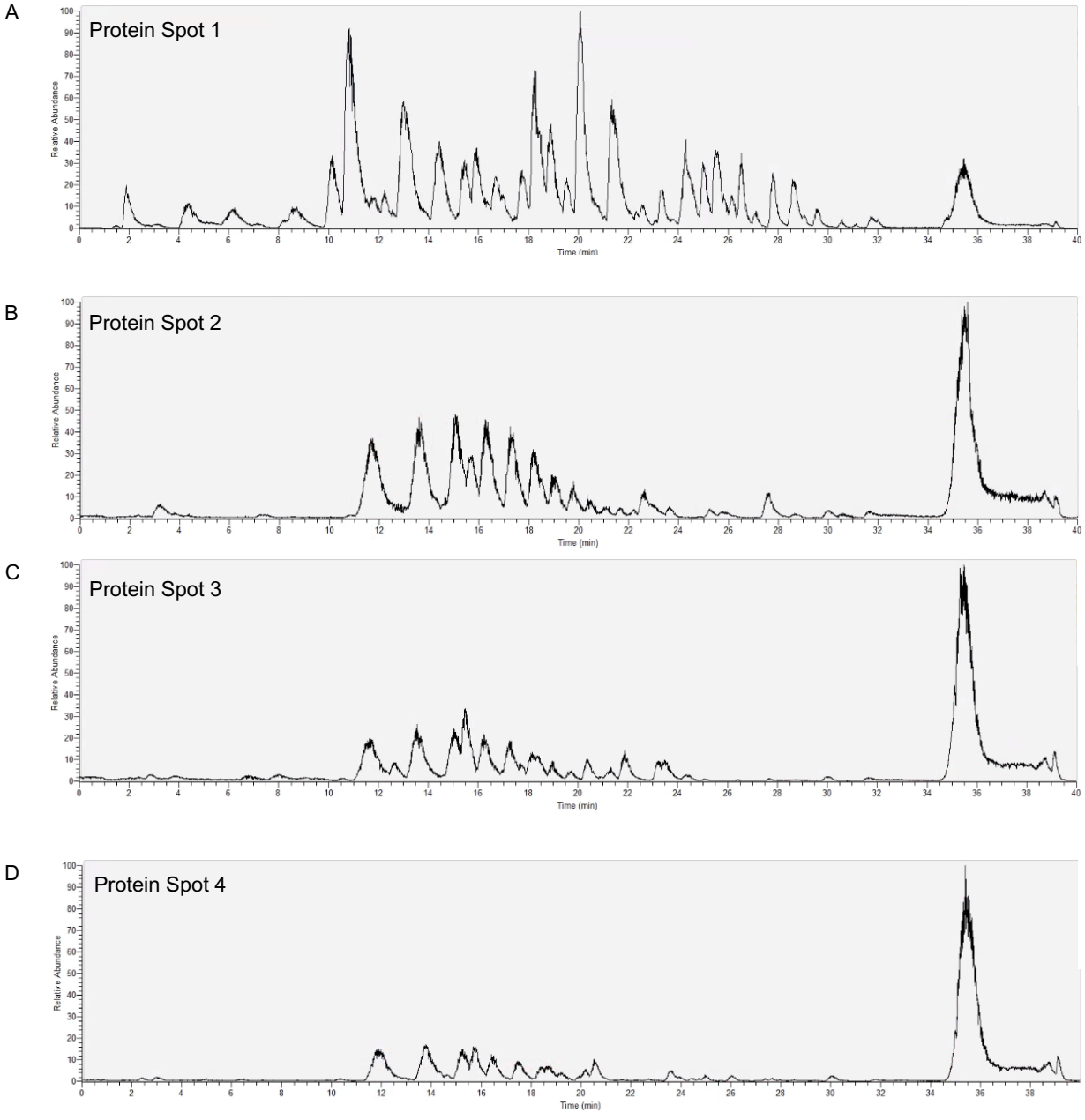
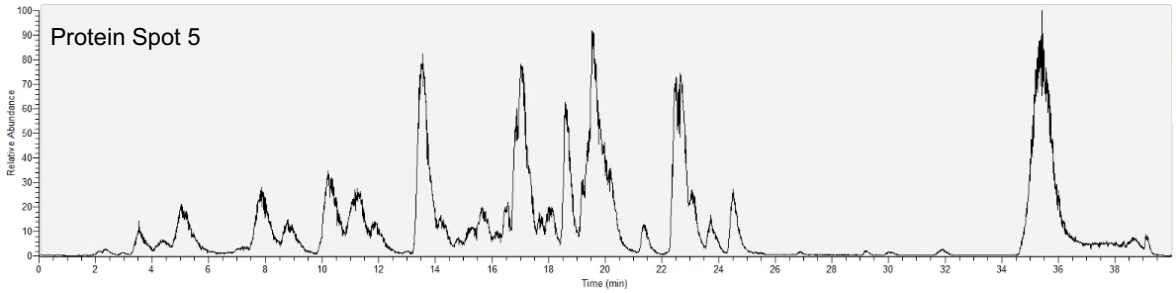


Figure 6.S3. 2-D gel images of protein samples isolated from *E. coli* culture treated with microwave (M) and incubated in water bath (Control).

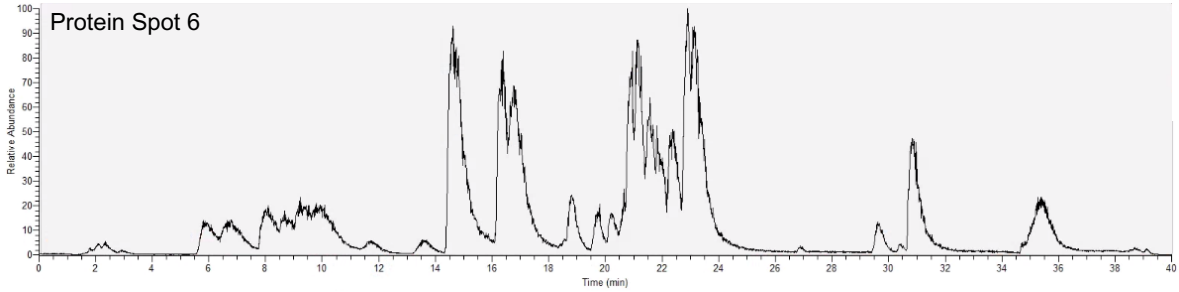
C = control, M = microwave, 100 µg total protein, 12.5% T mini-tall SDS-PAGE Loading and IEF order: Run 1 (C2-ABC; M2-ABC); Run 2 (C3-ABC; C4-ABC; M3-ABC; M4-ABC). 2, 3 and 4 represent biological replicates; A, B and C are technical replicates. For SDS-PAGE, a single gel was shared between corresponding strips, i.e. C2A M2A were run together. C2 and M2 were run on gels cast in parallel; C3, C4, M3 and M4 were run on gels cast in parallel.



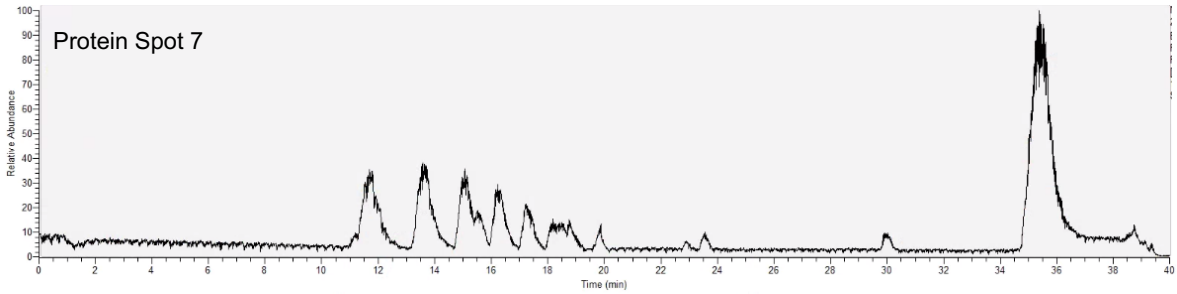
E


Protein Spot 5
Relative Abundance
Time (min)

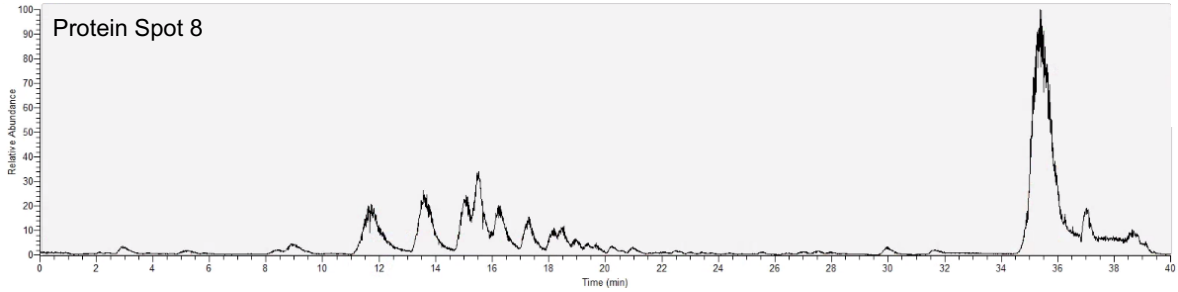
F


Protein Spot 6
Relative Abundance
Time (min)

G


Protein Spot 7
Relative Abundance
Time (min)

H


Protein Spot 8
Relative Abundance
Time (min)

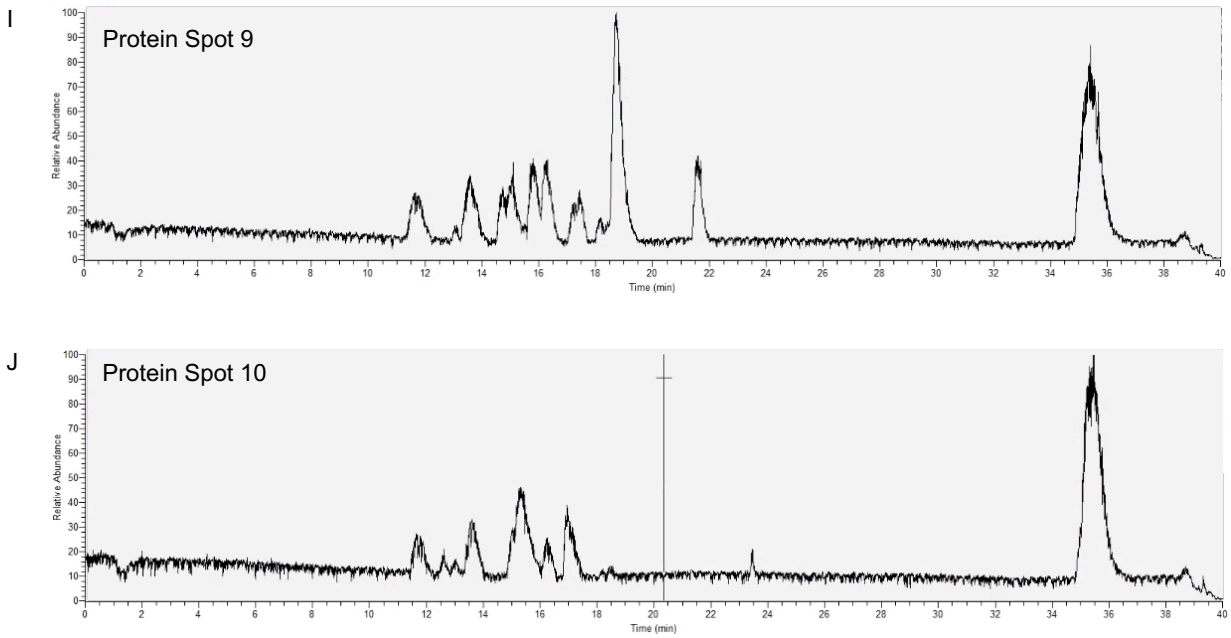


Figure 6.S4. LC-MS chromatograms of the 10 resolved protein spots. Chromatographic separation of peptides was carried out using a Zorbax 300SB-C18 column (3.5 µm i.d. × 150 mm, particle size 5 µm, pore size 100 Å, Agilent Technologies, Wilminton, DE). Peptides were loaded onto a Zorbax 300SB-C18 trap cartridge at a flow rate of 2 µl per minute for 10 min. After washing with 0.1% formic acid the peptides were eluted using a 5–40% B gradient for 30 min at a flow rate of 250 nl/min acid (mobile phase A = 0.1% formic acid; mobile phase B = 0.1% formic acid in acetonitrile) on an Agilent 1260 capillary/nano system.

Chapter 7

Summary and Conclusion

Experiments reported in this thesis studied the effects of microwave irradiation (2.45 GHz, power output not more than 10 W {In some experiments of chapter 2, power output of maximum 20 W was used}) on biomolecules and biological systems. Key factors considered throughout the experiments were microwave power output, temperature regulation, and mixing of media. Statistical analysis was implemented to ensure the statistical relevance of the results.

Microwave power density

All the experiments described in this thesis were carried out in a CEM Discover microwave reactor, which generate microwave using a circular magnetron. According to manufacturer's specifications, the power density in the cavity at power output of 10 W is 700 mW/ml. It is worth noting that when 2 mL of water is subjected to 10 W microwave irradiation in the current CEM setup, but without circulation of coolant, the water temperature rises from room temperature to 40°C in less than 1.5 minutes. Furthermore, temperatures measured by fibre optic temperature probes were found to be consistent with those measured by alcohol-based thermometer and thermocouple.

Maintenance of temperature and mixing homogeneity by stirring

To ensure thermal and mixing homogeneity a stirring bar was utilized in the reaction vessels in all of the experiments conducted. Lack of efficient stirring may lead to the generation of temperature gradient or the creation of hotspots due to the inherent inhomogeneities of MW EM fields. It is possible to improve the mixing efficiency by

increasing the stirring speed in the reaction vessel. Therefore, when appropriate, highest stirring speed by the machine (800 rpm) was used. In experiments on mammalian cells, the stirring speed was reduced to 540 rpm to reduce the mechanical stress on the cells. However, some studies have found the mixing efficiency of the standard stirring bars to be less than ideal in microwave reaction vessels. Obermayer et al. reported that the standard stirring bars are not always efficient in properly mixing the reactants in the microwave reaction vessels (which typically have high aspect ratio of height to diameter of the vessel), and this problem is more prominent with highly viscous or heterogeneous reaction mixtures. The authors found the efficiency of mixing to be less than optimal in the upper parts of the reaction vessels (specially with larger volumes) which could lead to the development of temperature gradient within the vessel (Obermayer et al., 2013).

Statistical analysis

To determine the statistical significance of the difference between the MW-irradiated and the control groups, two-tailed Student's t-Tests with the assumption of unequal variance were carried out on all biological replicates (MW & Ctrl) in Microsoft Excel. Experiments with p values not greater than 0.05 were considered as statistically significant.

A Welch's t-Test (only in chapter 6) for all protein spots of MW-treated and control samples was also carried out as described in the Materials and methods of chapter 6.

Summary of work

While it would have been ideal to establish the consequences of microwave irradiation in a dosage-dependent manner, this prospect of experiments is limited by the ability to keep

temperature constant (at higher microwave energy density) and to ensure reliable energy output at the lower end of energy densities. Nevertheless, efforts were made in some experiments in this study, *e.g*. internalization of doxorubicin by PC-3 and MCF-7 cells, where effects of microwave exposure were examined in a time-dependent manner. Work described in this thesis studied the potential influence of microwave irradiation on the activities of trypsin, α-amylase and alkaline phosphatase, membrane properties of human cancer cells, and *E. coli* growth, when microwave power output is kept up to 10 W, while temperatures of the systems were maintained virtually constant through cooling. Compared with similar studies reported in the literature, the present work focuses on the use of relatively low microwave power (up to 10 W), measurement of temperatures within the system in a real-time manner, and careful controlling of temperature through cooling. These considerations are of importance in order to potentially probe consequences of microwave exposure that are unique to this form of energy input.

In chapter 2, the influence of microwave irradiation on trypsin activity was explored using a CEM Coolmate apparatus at constant bulk temperature. Digestion of *N*α(±)-benzoyl-D/L-arginine-4-nitroanilide hydrochloride, azocasein and casein catalyzed by trypsin from bovine pancreas was found to be accelerated when the reaction mixture was exposed to microwave radiation, while the bulk temperature was maintained relatively constant through external cooling. Circular dichroism measurement of trypsin exposed to microwave irradiation suggested that there were changes in the secondary structure of trypsin exposed to microwave and conventional heating (through conduction), however, these changes were presumably due to self-cleavages of trypsin.

In chapter 3, we found that the acceleration of enzymatic reactions by microwave at constant bulk temperature is enzyme-dependent. While exposure of trypsin and its substrate to low power microwave, when the bulk temperature was kept constant, lead to significant increases of the activity of trypsin, the enzymatic activity of α-amylase and alkaline phosphatase towards the hydrolysis of starch and 4-nitrophenyl phosphate was not affected by exposure to microwave (10 W), with the reaction temperature maintained relatively constant.

In chapter 4, the impact of 10 W microwave irradiation at 2.45 GHz on the cell viability and membrane properties of PC3 human prostate cancer cells was investigated, while the temperature of the cell cultures was maintained at 37±2 ºC through simultaneous cooling. Microwave irradiation under these conditions did not induce apoptosis, nor otherwise lead to loss of cell viability, as was indicated by MTT, Annexin–V, and propidium iodide assays. Interestingly however, incorporation of a BODIPY fatty acid (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid) into the cell membrane was facilitated by microwave treatment at constant temperature. Also, microwave treatment of PC3 cells at constant temperatures significantly increased the rate of reduction of MTT. Taken together, these results suggested that irradiation of PC3 cells with microwave at constant temperature, while non-lethal to these cells, may alter membrane properties.

In chapter 5, exposure of MCF-7 breast and PC-3 prostate cancer cells to 10 W microwave at 2.45 GHz increased their uptake of the cancer drug doxorubicin (26 μM)

from media by almost 100% (based on median fluorescence count), concomitantly increasing cell death. However, microwave exposure alone had no cellular toxicity. The results suggested that targeted low power microwave could be an effective means of promoting delivery of chemotoxin to cancer cells.

In chapter 6, we examined the "microwave-specific effects" on bacterial growth. It was found that the growth of *E. coli* DE3 in LB media was slowed down in the presence of microwave at 2.45 GHz with a maximal power of 10 W, while the temperature of cultures was maintained at 37 °C (±2 °C) throughout the incubation via simultaneous cooling, compared with cultures that were not exposed to microwave. Viable cell counts in cultures irradiated with microwave were found to be lower as well. Measurement of the dissolved oxygen (DO) level in LB media revealed that the DO levels in media exposed to microwave irradiation were slightly lower than controls. When microwave irradiation was removed, *E. coli* growth was restored. The possibility was recognized that the slower growth of *E. coli* exposed to microwave irradiation at constant bulk culture temperature could be as a consequence of lower DO levels, accumulation of heat within cells, and adaptation to microwave irradiation as an external stress. While work is still ongoing, total proteins resolved by two-dimensional gel electrophoresis (2DE), followed by mass spectrometry (MS) analysis revealed that exposure of *E. coli* to MW under the conditions described in chapter 6 likely leads to the alteration of the proteomic profiles of the bacteria.

Overall, the results obtained in this thesis suggest that MW irradiation at constant bulk temperature can affect biological systems in a way that is significantly different from

what is observed under conventional heating (through conduction) methods at the same bulk temperature. The specific impact of MW was observed in certain biological systems and was absent in some others. However, the results do not necessarily suggest that the observed impacts are of non-thermal nature. The possibility that selective heating could be the cause of the observed microwave-specific effects in the study, cannot be ruled out.

Future work

Future studies on the effect of MW irradiation on enzymatic activity of trypsin, amylase and phosphatase should investigate the effect at other frequencies (above and below 2.45 GHz) and power intensities (above and below 10 W). These studies will provide further information on whether the effect of MW irradiation on enzymatic activity is power/frequency dependent and/or enzyme-specific. Also, the effect on other enzymes should be investigated. From mechanistic point of view, microwave absorption spectroscopy of enzymes would provide insight into possible "resonance" of enzymes with microwave, which could be useful in the interpretation of the effects reported in this thesis. Also, the potential effect of MW irradiation on enzyme structures in real-time should be investigated. For this purpose, a custom-built instrument capable of real-time CD spectroscopy of the reactants under MW irradiation is required.

The effect of MW irradiation on the cell membrane fluidity or permeability should be further investigated. Future studies should investigate the MW effect on cell membrane at the molecular level and elucidate the mechanisms involved in the increased permeability

observed in this thesis which are currently not known. Studies in chapter 6 suggested that the uptake of doxorubicin by cancer cells can be enhanced under MW irradiation. However, to further explore the potential application of MW irradiation as a drug delivery system, *in vivo* studies are required. A study by Maeda et al. demonstrated the possibility of *in vivo* optical imaging of subcutaneous xenograft tumors by confocal microscopy, using surgically implanted window chambers on a mouse dorsal skinfold (Maeda et al., 2012). A similar approach can be used to investigate the effect of local microwave irradiation and doxorubicin on such tumors. Towards this end, doxorubicin would be introduced to mice intravenously. The subcutaneous xenograft tumors will be treated with or without local MW irradiation, and the growth and fluorescence of tumors will be analyzed by confocal laser scanning microscopy over the period of study. Another interesting study would be to compare the uptake of compounds by cells under MW irradiation at 2.45 GHz to electroporation. For this purpose, fluorescent silica nanospheres of various sizes (e.g. 10-70 nm in diameter) can be used. The uptake of the nanospheres by cells can then be assessed using techniques such as flow cytometry or confocal laser scanning microscopy. The maximum size of the nanospheres that can be taken up under either treatment can then be compared. Nguyen et al. showed that the uptake of fluorescent nanospheres with diameters of 46.3 nm could occur by various cell types (eukaryotic and prokaryotic) under MW irradiation at 18 GHz (SAR: 3.0 - 5.0 kW/kg) (Nguyen et al., 2015; Nguyen et al., 2017). In addition, the potential role of cell membrane as a heat exchange barrier should be investigated. Developing methods to measure the temperature in the intracellular space under MW irradiation and comparing it

to the temperature in the inter-cellular space or the temperature of the bulk solution would be necessary for this purpose.

While the present work on proteomic analysis has revealed that exposure of *E. coli* to non-lethal microwave irradiation led to changes in the level of expression of a range of proteins involved in metabolism, it will be interest to validate the metabolic pathways affected by microwave irradiation. Furthermore, future work should extend this proteomic approach to study mammalian cells as well. While not reported in this thesis, work has started to investigate the influence of similar non-lethal microwave irradiation on the transcription of DNA in *E. coli* through RNA sequencing. In this respect, total RNA samples were extracted from three biological replicates of control and microwave-treated *E. coli* cultures and then subjected to RNA sequencing after ribosomal RNA depletion on an Illumina NextSeq platform. Work is currently in progress to analyze the RNA-seq data through bioinformatics, and to validate the affected transcripts by quantitative PCR.

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